UNIVERSITAT DE BARCELONA

Mecanismos moleculares que confieren resistencia a la apoptosis por TGF-β en células de Hepatocarcinoma Celular Humano

Laia Caja Puigsubirà

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"Mecanismos moleculares que confieren resistencia a la apoptosis por TGF-β en células de Hepatocarcinoma Celular Humano"

Memoria presentada por

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para aspirar al

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ABBREVIATIONS

4E-BP1	Eukaryotic Initiation Factor 4E-Binding Protein-1
A1AT	Alpha1-Antitrypsin
ADAM17	A Disintegrin And Metalloprotease 17
AFP	Alfa-Fetoprotein
AGM	Aorta–Gonads–Mesonephros
ALB	Albumin
ALK	Activin Receptor-Like Kinase
ANG II	Angiotensin II
AP1	Activating Protein-1
APCs	Antigen-Presenting Cells
AR	Amphiregulin
ASC	Ascorbic Acid
ASK1	Apoptotic Signal-Regulating Kinase 1
BCLC	Barcelona-Clinic Liver Cancer
BEC	Biliary Epithelial Cells
BH	Bcl-2 Homology
bHLH	Basic Helix–Loop–Helix
BIR	Baculovirus Iap Repeat
BMP	Bone Morphogenic Protein
BTC	Betacellulin
C/EBP	CCAAT/Enhancer Binding Proteins
CARD	Caspase Activation And Recruitment Domain
CB	Cathepsin B
CC	Cholangiocarcinomas
CD	Cathepsin D
CDH1	E-Cadherin
CDH2	N-Cahderin
CDKs	Cyclin-Dependent Kinases
CK	Citokeratin
CL	Cathepsin L
CSC	Cancer Stem Cells
CTGF	Connective-Tissue Growth Factor
CTLs	Cytotoxic T Lymphocyte
CTP	Child–Turcotte–Pugh
Cx	Connexin
DD	Death Domain
DED	Death-Effector Domain
DISC	Death-Inducing Signaling Complex
DPI	Diphenylene Iodonium
E	Embryonic Day
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition

eNOS	Endothelial Nitric Oxide Synthase
EpCAM	Epithelial Cell Adhesion Molecule
EPG	Epigen
EPR	Epiregulin
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
ES	Embryonic Stem Cells
FAD	Flavin Adenine Dinucleotide
FGF	Fibroblast Growth Factor
FSP1	Fibroblast-specific protein 1
GEE	Glutathione-Ethyl-Ester
γ-GCS	gamma-Glutamylcysteine Synthetase
GPCRs	G Protein-Coupled Receptors
Grb2	Growth-Factor-Receptor Bound-2
GSH	Glutathione
GSSG	Oxidized Glutathione
H_2O_2	Hydrogen Peroxide
HB	Hepatoblast
HB-EGF	Heparin Binding-Epidermal Growth Factor –like growth factor
HBV	Hepatitis B Virus
HCC	Heptacolellular Carcinoma
HCV	Hepatitis C Virus
HDACs	Histone Deacetylases
HFH	Human Fetal Hepatocytes
HGF	Hepatocyte Growth Factor
hHpSCs	Human Hepatic Stem Cells
HNF	Hepatocyte Nuclear Factor
HO-1	Hemoxygenase
HOCl	Hypochlorous Acid
HPC	Hepatic Progenitor Cells
HSC	Hepatic Stellate Cells
IAP	Inhibitor-of-Apoptosis Protein
IBM	IAP-Binding Motif
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IGF	Insulin-Like Growth Factor
IGFBP	IGF Binding Protein
IHBD	Intrahepatic Bile Ducts
IL	Interleukin
iPS	Pluripotent Stem Cells
JAK	Janus Kinases
JNK	C-Jun N-Terminal Kinase
LIF	Leukemia Inhibitory Factor
LMP	Lysosomal Membrane Permeabilization
LPA	Lysophosphatidic Acid
LRIG1	Leucine-Rich repeats and Immunoglobulin-Like Domains-1

LTBPs	Latent TGF-B Binding Proteins
M2PK	M2 Isoform of Pyruvate Kinase
MAPK	Mitogen-Activated Protein Kinase
MET	Mesenchymal–Epithelial Transition
MDCK	Madin-Darby canine kidney cells
MH	Mad Homology
miRNAs	MicroRNAs
MMP	Metalloprotease
MOMP	Mitochondrial Outer-Membrane Permeabilization
MPO	Myeloperoxidase
MSCs	Mesenchymal Stem Cells
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NCAM	Neuronal Cell Adhesion Molecule
NEDD4-2	Neuronal Precursor Cells Expressed, Developmentally Downregulated 4-2
NF-κB	Nuclear Factor-Kappab
NLS	Nuclear Localization Signal
NO·	Nitric Oxide
NOX	NAPDH Oxidase
NOXA1	Nox Activator Protein 1
NOXO1	Nox Organizer Protein 1
NRGs	Neuregulins
NSCLC	Non-Small Cell Lung Cancer
O_2· -	Superoxide anion
OC	Onecut
OH·	Hydroxyl Anion
OONO-	Peroxynitrite
OSM	Oncostatin M
p70s6K	Protein S6 Kinase
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
PDK-1	Phosphoinositide-Dependent Kinase-1
PDP	Pyruvate Dehydrogenase Phosphatase
PDTC	Pyrrolidine Dithiocarbamate
phox	Phagocytic Oxidase
PI3K	Phosphoinositide-3-Kinase
PIAS	Protein Inhibitors of Activated STAT
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PIP3	Phosphatidylinositol 3,4,5-Trisphosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
PLCc	Phospholipase Cc
PLD	Phospholipase D
PtdIns	Phosphatidylinositols
PTEN	Phosphatase and Tensin Homologue
PTP1B	Protein Tyrosine Phosphatase-1B

RIP	Receptor-Interacting Protein
RISC	RNA-Induced Silencing Complex
ROS	Reactive Oxygen Species
RCT	Positive Randomized Controlled Trial
RTKs	Receptor Tyrosine Kinase
RT-MLPA	Reverse Transcriptase Multiple Ligation Dependent Probe Amplification
SAPK	Stress-Activated Protein Kinase
SBE	Smad Binding Element
SEC	Sinusoidal Endothelial Cells
SHC	Src-homology-2-containing
SMA	α-Smooth Muscle Actin
Smurf	Smad Ubiquitination Regulatory Factors
SNAI1	Snail
SOCS	Suppressors Of Cytokine Signaling
SOD	Superoxide Dismutase
SOH	Sulfenic Acid
SOS	Son Of Sevenless
SP	Side Population
SPRY	Sprouty
STAT	Signal Transducers and Activators of Transcription
SO2H	Sulfinic Acid
SO3H	Sulfonic Acid
SSR	Disulfide Bond
SUMO	Small Ubiquitin-Like Modifier
TACE	Transarterial Chemoembolization
TACE	TNF-Alpha Converting Enzyme
TAK1	TGF-beta-Activated Kinase 1
TβRI	TGF-beta Receptor I
TβRII	TGF-beta Receptor II
TβT-HFH	TGF-beta-Treated Human Fetal Hepatocytes
TF	Transcription Factor
TGF-α	Transforming Growth Factor-Alpha
TGF-β	Transforming Growth Factor-Beta
TIMP	Tissue Inhibitors of Metalloproteinases
TKs	Tyrosine Kinases
TLR	Toll Like Receptors
TNF-α	Tumor Necrosis Factor-Alpha
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRAIL-R	TNF-Related Apoptosis-Inducing Ligand Receptor
Trx	Thioredoxin
UCB	Umbilical Cord Blood
UPR	Unfolded Protein Response
UTRs	Untranslated Regions
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VIM	Vimentin

VSMCs Vascular Smooth Muscle Cells

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I. SUMMARY

In the last years our research has focused on analyzing the signaling pathways induced by TGF-β in liver tumor cell lines, to understand the molecular mechanisms that confer resistance to its suppressor effects. TGF-β induces apoptosis in human fetal hepatocytes and in some liver tumor cells (FaO rat hepatoma, Hep3B and PLC/PRF/5 human hepatocarcinoma cells), which requires reactive oxygen species (ROS) production and up-regulation of the NADPH oxidase NOX4. This process is coincident with an increased expression of pro-apoptotic BCL-2 family members, such as BMF or BIM. However, in these same cells, TGF-β also induces anti-apoptotic signals, mediated by the activation of the epidermal growth factor receptor (EGFR) and coincident with up-regulation of the anti-apoptotic proteins BCL-XL, MCL1 or HIAP1. Inhibition of the EGFR, either by pharmacological inhibitors or through targeting knock-down with specific siRNA, significantly enhances the apoptotic response, which indicates that the EGFR plays a relevant role in conferring resistance to TGF-β-induced cell death. However, even when the EGFR is inhibited, some hepatocellular carcinoma cells, such as HepG2 or SK-Hep1, continue showing resistance to TGF-β-induced cell death. HepG2 cells are sensitized to TGF-β-induced apoptosis through the inhibition of the MEK pathway. MEK inhibition allows TGF-β to induce its pro-apoptotic program in these cells, which is coincident with NOX4 upregulation, modulation of the expression of BCL-2 family members and caspase-3 activation. It is worthy to note that activation of survival pathways, such as EGFR or MEK/ERK, in liver tumor cells confers resistance to TGF-β-induced cell death through impairing NOX4 up-regulation, which is required for an efficient mitochondrial-dependent apoptosis. Finally, our results have indicated that TGF-β is able to induce an epithelial to mesenchymal transition (EMT) process in human fetal hepatocytes, FaO rat hepatoma cells and Hep3B human hepatocarcinoma cells. TGF-β induces Snail expression, coincident with a decrease in E-cadherin mRNA and protein levels. Furthermore, cells show an increased expression of mesenchymal genes and reorganization of the actin cytoskeleton in stress fibers. Interestingly, these cells show loss of expression of specific hepatic markers and increased expression of stem cell markers. Indeed, chronic treatment with TGF-β selects a population of mesenchymal cells with a de-differentiated phenotype, reminiscent of progenitor-like cells. In summary, TGF-β induces different signals in liver tumor cells, some of them might contribute to tumor suppression (apoptosis), but others should mediate liver tumor progression and invasion.

II. RESUMEN EN ESPAÑOL

Introducción

El hígado y el carcinoma hepatocelular

El **hígado** es el mayor órgano interno en mamíferos, cuyas principales funciones son: 1) la producción de proteínas del suero; 2) eliminación de proteínas del suero, de glóbulos rojos y patógenos; 3) producción o eliminación de glucosa en períodos de ayuno o ingesta; 4) procesamiento de ácidos grasos y triglicéridos; 5) mantenimiento de la homeostasis del colesterol; 6) síntesis de aminoácidos no esenciales; 7) eliminación de compuestos endógenos tóxicos; 8) producción y excreción de componentes de la bilis; 9) detoxificación de agentes xenobióticos; y 10) almacenamiento de varias sustancias como vitaminas (Costa et al. 2003; Malarkey et al. 2005; Spear et al. 2006).

El hígado está formado por varias unidades de forma "hexagonal", denominadas lóbulos, que están delimitadas en el centro por la vénula hepática. En los extremos se encuentra la tríada portal, que contiene la vena portal, la arteria hepática y los ductos biliares. En el espacio portal, los segmentos terminales del sistema biliar conectan con los hepatocitos en el parénquima hepático a través de los canales de Hering (Fausto and Campbell 2003; Spear et al. 2006). En el ser humano el hígado se divide en 4 lóbulos, denominados derecho, izquierdo, cuadrado y caudado (Malarkey et al. 2005). Las células que se encuentran en los canales de Hering en el hígado adulto son células madre o células progenitoras, denominadas células ovales en ratones/rata y hepatoblastos en humanos. Estas células expresan marcadores tanto de hepatocitos como de células biliares y son capaces de generar ambos tipos celulares (Fausto and Campbell 2003; Spear et al. 2006). El hígado adulto está formado por varios tipos celulares aparte de los hepatocitos, de ellos los mayoritarios son: células epiteliales del ducto biliar, células ovales, células de Kupffer, células estrelladas, células endoteliales sinusoidales y células Pit (Guyton and Hall 1999; Malarkey et al. 2005).

En el desarrollo del hígado se pueden distinguir varias etapas. En una primera fase ocurre la especificación, donde las células adquieren competencia y son capaces de adquirir un destino celular, aunque no se observa ningún cambio morfológico. Estas células competentes se "comprometen" a un linaje celular mostrando cambios morfológicos y la expresión de genes asociados a ese "compromiso". Entonces las células se diferencian (Spear et al. 2006). Para el desarrollo del hígado es necesaria la liberación de varias citoquinas desde las capas embrionarias adyacentes, tales como el mesodermo cardíaco y la región del septum transversum, que liberan FGF y BMP4, que, a través del factor de transcripción GATA-4, contribuyen a la "competencia" y especificación del endodermo pre-hepático (Rossi et al. 2001; Huang et al. 2008). Otras citoquinas y factores que también tienen una función importante en el desarrollo del hígado son el HGF, el TGF-β, glucocorticoides y Oncostatina M, igual que la vía Wnt/β-catenin (Lemaigre 2009). No sólo la expresión de GATA-4 es imprescindible para el desarrollo, sino que otros factores de transcripción son de gran importancia: 1) GATA-6; 2) la familia Foxa (Forkhead box a) formada por tres miembros Foxa1, Foxa2 y Foxa3 (también conocidos como HNF3α, HNF3β y HNF3γ); 3) la familia ONECUT, compuesta por HNF6/ONECUT-1(OC-1) y OC-2; 4) HNF1α y HNF1β, 5) HNF4α, y 6) C/EBPα y C/EBPβ (Lemaigre and Zaret 2004). Brevemente, HNF6 y OC-2 controlan la diferenciación de los hepatoblastos hacia el linaje hepatocítico, en cambio el HNF1β favorece el desarrollo de los ductos biliares intrahepáticos. Los factores HNF1α/β, c/EBPα, HNF4α, y Foxa actúan conjuntamente para controlar la diferenciación de los hepatocitos y la función hepática. Entre otras funciones, los factores GATA-4 y GATA-6 regulan la expresión de albúmina y HNF4α (Spear et al. 2006). Además, la expresión de HNF4α es necesaria para mantener el fenotipo diferenciado de los hepatocitos en el hígado adulto (Lemaigre and Zaret 2004; Zhao and Duncan 2005).

En el hígado humano se han descrito dos tipos de células progenitoras pluripotentes: células madre hepáticas (hHpSCs) y hepatoblastos/células ovales (Zhang et al. 2008). Las hHpSCs dan lugar a los hepatoblastos, que a su vez se pueden diferenciar en hepatocitos o células biliares. El porcentaje de hepatoblastos en el hígado varía a lo largo del desarrollo. Es mayoritario en hígados fetales y neonatales y pasa a ser menos de 0.1% en hígados pediátricos y adultos, donde las células mayoritarias son los hepatocitos (Schmelzer et al. 2006). Las células ovales son pequeñas células epiteliales con el núcleo oval y poco citoplasma (Libbrecht and Roskams 2002). Estas células expresan proteínas características de los dos linajes a los que pueden diferenciarse, ya que expresan marcadores hepáticos (AFP, Albúmina, CK8/18) y marcadores de célula biliar (CK7/19), además son positivas para el marcador OV6, y expresan varios marcadores típicos de célula madre hematopoyética (c-Kit, CD34, CD90/Thy1, LIF y Sca-1) (Roskams et al. 2003; Kakinuma et al. 2009). Diferentes grupos han descrito marcadores característicos de hHpSCs. Por ejemplo, se ha descrito que tienen capacidad de auto-renovación, y presentan el siguiente inmuno-fenotipo: CD34+, CD90+, c-kit+, EpCAM+, c-met+, SSEA-4+, CK18+, CK19+, albúmina-, α-fetoproteina-, CD44h+, y vimentina+. Estas células pueden diferenciarse tanto hacia hepatocitos y células biliares, como hacia células de tipo mesenquimático (Dan et al. 2006). Otro grupo describe tanto a las HpSCs como a los hepatoblastos como células EpCAM+, diferenciándolas entre ellas porque las HpSCs son Albúmina-/AFP- y con niveles muy bajos de genes específicos del hígado (conexinas, PEPCK, DPP4, citocromo P450 3A4, y transferina), y los hepatoblastos expresan AFP (Schmelzer et al. 2006; Schmelzer et al. 2007).

En el hígado adulto, los hepatocitos tienen una tasa de crecimiento muy baja, únicamente relacionada con el mantenimiento del número de células para compensar la muerte fisiológica de las células. Sin embargo, después de una hepatectomía parcial los hepatocitos, y las células biliares (el componente epitelial), así como los diferentes tipos de células mesenquimales proliferan para recuperar la masa original del hígado. Si el componente epitelial es dañado gravemente se "activan" las células progenitoras, que proliferan y se diferencian (Roskams 2006; Weiss et al. 2008). Este proceso ocurre a diferentes niveles en la mayoría de enfermedades hepáticas (Libbrecht and Roskams 2002).

El carcinoma **hepatocelular** (HCC) es el quinto cáncer más común en el mundo, y la tercera causa de muerte entre los diferentes tipos de cánceres (Llovet and Bruix 2008). La mayoría de HCC derivan de un proceso de fibrosis y cirrótico, siendo los principales factores de riesgo las infecciones crónicas de virus de hepatitis B o C, alcoholismo, y enfermedades metabólicas del hígado (Alison and Lovell 2005; Llovet and Bruix 2008).

Hay dos teorías principales sobre el origen del HCC. Por un lado, se cree que es debido a la acumulación de mutaciones en genes críticos para la capacidad de auto-renovación, crecimiento y muerte celular en los hepatocitos adultos, que dará lugar a la célula cancerígena (Hahn and Weinberg 2002); por otro, se sugiere que se originan a partir de las células progenitoras hepáticas, que dan lugar a las denominadas células madre tumorales. Esta segunda teoría se basa en que más del 50% de los HCC expresan marcadores de células progenitoras como CK7, CK19, CK14 y AFP (Libbrecht and Roskams 2002), OV6, EpCam y c-Kit (Yang et al. 2008c), o CD133 (Ma et al. 2007b). Otra posibilidad es que los hepatocitos maduros, después del proceso de transformación, se desdiferencien y adquieran propiedades de célula madre (Libbrecht and Roskams 2002). Hay varios estudios que clasifican los tumores de HCC según su estado de diferenciación, según la expresión de EpCAM y presencia de AFP en sangre (Yamashita et al. 2008b), o bien según la expresión de marcadores de hepatoblastos fetales, como niveles elevados de CK7 y CK19 (Lee et al. 2006a). Ambos estudios asocian el fenotipo más desdiferenciado con un peor pronóstico.

En la actualidad, no hay un tratamiento efectivo para el HCC. El más efectivo es la resección o trasplante del hígado, pero sólo el 40% de los pacientes son elegidos para estos procesos, ya que la mayoría de casos se detectan en un estadio más avanzado y son inoperables. Gelfoam, doxorrubicina o cisplatino se utilizan como terapias que incrementan la supervivencia de los pacientes (Vander Borght et al. 2008; Yang et al. 2008d). Hasta el 2006 no había tratamiento para los pacientes en un estadio avanzado, aunque actualmente se usa el inhibidor multiquinasa Sorafenib (Llovet et al. 2008).

Se han observado varios mecanismos moleculares alterados en HCC, principalmente: 1) mecanismos de regulación del ciclo celular como p16INK4A, p21(WAF1/CIP1), p27KIP, o pRB (Azechi et al. 2001); 2) mutaciones en p53 (Hsu et al. 1991); 3) desregulación de varias vías de señalización que finalmente conducen a la evasión de la apoptosis (Fabregat 2009), como la sobre-expresión de diferentes proteínas anti-apoptóticas (BCL-XL, MCL1, XIAP, c-IAP1, c-IAP2); 4) activación de las vías Wnt/β-catenin (Aravalli et al. 2008), JAK/STAT (Calvisi et al. 2006), c-MET (Newell et al. 2008), IGF, PI3K (Llovet and Bruix 2008), RAS/Raf/ERK (Thomas 2009), y el EGFR (Lee et al. 2007c); 5) la desregulación de la expresión de varios microRNAs (Minguez et al. 2009); y 6) procesos de angiogénesis aberrantes (Tanaka and Arii 2009).

Apoptosis

Se han descrito diferentes tipos de muerte celular: apoptosis, necrosis, muerte independiente de caspasas, autofagia, permeabilización de la membrana lisosomal (LMP) o estrés de retículo endoplasmático (UPR). La **apoptosis** es un tipo de muerte celular programada, que va acompañada de un conjunto de cambios morfológicos característicos: el núcleo se condensa, la célula se encoge, pierde la asimetría de la membrana plasmática, los orgánulos también sufren un proceso de fragmentación, y finalmente aparecen burbujas en la superficie celular, formándose los cuerpos apoptóticos (Schattenberg et al. 2006; Taylor et al. 2008). La apoptosis puede ser iniciada a través de dos vías: la extrínseca, desencadenada por la unión de ligandos a receptores de muerte localizados en la membrana celular, y la intrínseca, mediada por la mitocondria y regulada por la familia BCL-2. En ambos casos las proteínas efectoras de la apoptosis son las caspasas (Giam et al. 2008; Dewson and Kluck 2009), y este proceso es regulado negativamente por la familia IAPs (inhibitors of apoptosis) (LaCasse et al. 2008).

Las caspasas son cisteín-aspartato proteasas que se sintetizan como zimógenos inactivos, y por tanto necesitan ser proteolizadas para activarse. Esta familia se divide en las caspasas iniciadoras (caspasa-1, -2, -4, -5, -8, -9, -10, -11, y -12), que responden a los estímulos pro-apoptóticos, y las efectoras (caspasa-3, -6, -7, y -14) (Yi and Yuan 2009). En general, las caspasas proteolizan sus sustratos en una o más posiciones, lo que conduce a la inactivación de la proteína diana; sin embargo, también hay casos en los cuales la proteolización promueve la activación de la proteína diana, como es el caso de la DNAsa CAD (Hengartner 2000).

La familia BCL-2 es responsable de la regulación de la vía intrínseca, y se activa en respuesta a diferentes estímulos de estrés. Estas proteínas se caracterizan por la presencia de uno o varios dominios BH (Frenzel et al. 2009). La familia se subdivide en tres grupos: 1) las proteínas anti-apoptóticas, formado por BCL-2, BCL-XL, BCL-w, MCL1 y A1/BFL-1; 2) proteínas pro-apoptóticas, que presentan 3 dominios BH: BAX, BAK y BOK; y 3) las proteínas pro-apoptóticas “BH3-only”, que se dividen a su vez en *activadoras* (BIM, BID y PUMA) y *desrepresoras* (BAD, BIK/NBK, NOXA, BMF, HRK/DP5, EGl-1, BNIP3, BNIP3L y BECLINA) (Certo et al. 2006; Lomonosova and Chinnadurai 2008). Los diferentes “BH3-only” forman complejos de unión con los miembros anti-apoptóticos de la familia de manera específica. Se han propuesto dos modelos de actuación de la

familia BCL-2. El modelo indirecto propone que en la situación basal de la célula todas las proteínas BAX y BAK deben estar secuestradas por los miembros anti-apoptóticos de la familia BCL-2; cuando la célula recibe un estímulo apoptótico los “BH3-only” se unen a las proteínas anti-apoptóticas, liberando así a BAX y BAK (Chen et al. 2005; Willis et al. 2005). El modelo directo divide las proteínas “BH3-only” en *activadoras* que pueden unirse y activar a BAX y BAK, además de unirse a los miembros anti-apoptóticos, y en *desrepresoras* (Letai et al. 2002; Kuwana et al. 2005). Según este modelo, en una célula en estado basal las proteínas anti-apoptóticas de la familia BCL-2 se unen a las “BH3-only” *activadoras*. Cuando la célula recibe un estímulo, las proteínas *desrepresoras* se unen a las proteínas anti-apoptóticas, liberando a las “BH3-only” *activadoras*, que a su vez promueven la activación de BAX y BAK (Cartron et al. 2004; Certo et al. 2006). También se ha propuesto que la iniciación de la vía intrínseca requiere aspectos de ambos modelos (Merino et al. 2009). Una vez activados, BAX y BAK oligomerizan y forman poros en la membrana externa de la mitocondria causando su permeabilización, y la liberación del contenido del espacio intermembrana mitocondrial, como el citocromo c, responsable de la activación de la caspasa-9.

Las proteínas IAPs tienen en común el dominio BIR (Baculovirus IAP Repeat). La familia está compuesta por 8 miembros: BIRC1/NAIP, BIRC2/cIAP1, BIRC3/cIAP2, BIRC4/XIAP, BIRC5/Survivin, BIRC6/Apollon/BRUCE, BIRC7/ML-IAP y BIRC8/ILP2 (Hunter et al. 2007). También presentan el dominio RING con función de ubiquitinización, que induce la degradación proteosomal de los sustratos sobre los que actúan, entre ellos las caspasas (Dubrez-Daloz et al. 2008).

De los otros tipos de muerte celular, merece la pena mencionar **la permeabilización de membrana lisosomal** (LMP). El desmantelamiento total de los lisosomas induce necrosis, pero un proceso parcial de LMP desencadena apoptosis. Algunas catepsinas, proteasas lisosomales, permanecen activas en pH neutro, actuando sobre la mitocondria o activando a las caspasas. El proceso de LMP puede ser inducido por p53 y receptores de muerte entre otros (Conus and Simon 2008). Además se ha observado que LMP puede inducir MOMP ya que diferentes catepsinas pueden activar a BID (Cirman et al. 2004); finalmente, también se ha observado que BIM y BAX se translocan al lisosoma induciendo LMP (Feldstein et al. 2004; Boya and Kroemer 2008).

Estrés oxidativo y NADPH oxidasas

Las especies reactivas de oxígeno (**ROS**) son un grupo de moléculas producidas por las células cuando el oxígeno es metabolizado. Las más relevantes desde un punto de vista fisiopatológico son: anión superóxido (O_2.-), óxido nítrico (NO·), peróxido de hidrógeno (H_2O_2) y radical hidroxilo (·OH). Las ROS, y en particular O_2.-, son producidas por varias enzimas: NADPH oxidasas (Nox), ciclooxigenasas y xantina oxidasa, entre otras. Si la concentración de H_2O_2 es elevada, estos procesos oxidativos pueden causar un daño irreversible seguido de la muerte celular, pero a bajas concentraciones las ROS son capaces de regular la función de diferentes enzimas, entre ellas las fosfatasas, y modular vías de señalización intracelular (Brown and Griendling 2009; Groeger et al. 2009). Además, la célula dispone de diversos mecanismos antioxidantes que la protegen del daño oxidativo inducido por la producción de ROS. Así, diferentes enzimas, como las superóxido dismutasas, la catalasa, las peroxiredoxinas, o la glutation peroxidasa, metabolizan las ROS, contribuyendo a disminuir sus concentraciones intracelulares. Tioredoxinas y hemoxigenasa tienen también una elevada importancia al proteger a las proteínas de daños oxidativos. Pero, sin lugar a dudas, la molécula clave en la protección celular frente al daño oxidativo es el tripéptido glutatión, cuya forma reducida (GSH) aporta poder reductor interviniendo en un gran número de las reacciones catalizadas por las enzima antioxidantes y protegiendo el estado reducido de los grupos sulfhidrilo de las proteínas (Brown and Griendling 2009; Chen et al. 2009). La enzima reguladora en la síntesis de

glutatión es la γ-glutamil-cisteina, cuya expresión aumenta en condiciones fisiológicas cuando la célula necesita aumentar la síntesis de este tripéptido.

Los miembros de la **familia de NADPH oxidasas**, Nox, son proteínas que transfieren electrones a través de membranas biológicas. En general, el aceptor de electrones es el oxígeno y el producto de la reacción es el anión superóxido. La función biológica, por tanto, de las enzimas Nox es la generación de especies reactivas de oxígeno (Brown and Griendling 2009). El miembro más conocido es Nox2, o gp91phox, que se expresa en células fagocíticas. A partir de 1999 se han descubierto 6 miembros más de esta familia: Nox1, Nox3, Nox4, Nox5, Duox1 y Duox2 (Lambeth 2004; Groeger et al. 2009). Se clasifican en tres grupos según la presencia de diferentes dominios. Todas las isoenzimas presentan el domino Nox flavocitocromo, el dominio FAD, y cuatro histidinas de unión al grupo hemo. El primer grupo, formado por Nox1, Nox2, Nox3, y Nox4 no presentan ningún dominio adicional. El segundo grupo, donde se encuadra Nox5, presenta en el amino terminal un dominio de semejanza a calmodulina, por lo que su activación es dependiente de calcio. Finalmente el tercer grupo, formado por Duox1 y Duox2, presentan sitios de unión a calcio, pero además poseen un dominio extracelular con actividad peroxidasa (Lambeth et al. 2007; Chen et al. 2009; Groeger et al. 2009).

Para ser activa Nox2 necesita de la formación de un complejo multienzimático, el cual está formado por las siguientes subunidades: p22phox, p47phox, p67phox, p40phox, y la GTPasa pequeña Rac1/2. Otras Noxes, comparten varias de las subunidades co-activadoras, pero también pueden usar sus homologos Noxo1 (Nox organizer protein 1) y Noxa1 (Nox activator protein 1) (Lambeth et al. 2007; Chen et al. 2009; Oakley et al. 2009). La excepción es Nox4, cuya actividad depende fundamentalmente de sus niveles de expresión y que sólo necesita de la subunidad p22phox para incrementar su actividad (Krause 2004).

Las ROS producidas por **Nox4** se han implicado en varios procesos fisiológicos, incluyendo senescencia, apoptosis, supervivencia, migración, estrés del retículo endoplasmático y diferenciación (Brown and Griendling 2009). Se ha observado que tanto insulina en adipocitos (Schroder et al. 2009) como IGF-1 en VSMCs (Meng et al. 2008) inducen la expresión de Nox4, y que Nox4 es responsable de la inactivación por oxidación de PTP1B, incrementando la actividad de varias quinasas (Mahadev et al. 2004). También se ha descrito que Nox4 promueve la supervivencia y la proliferación en diferentes tipos celulares: por ejemplo, Nox4 induce crecimiento y supervivencia en VMSCs en respuesta a uPa (Menshikov et al. 2006); Nox4 está implicado en mediar proliferación de fibroblastos pulmonares adventiciales en respuesta a hipoxia (Li et al. 2008); en células pancreáticas, Nox4 media señales anti-apoptóticas (Vaquero et al. 2004) e induce la activación de la vía JAK/STAT (Lee et al. 2007a); en diferentes tipos celulares, y en respuesta a diferentes estímulos, se ha observado que Nox4 induce la activación de ERKs (Gorin et al. 2004; Amara et al. 2007; Datla et al. 2007; Wagner et al. 2007). Adicionalmente, Nox4 está implicado en la diferenciación de células madre embrionarias en ratón (Li et al. 2006a) o la diferenciación de fibroblastos en miocitos (Cucoranu et al. 2005). Finalmente, destacar que el TGF-β induce la expresión de Nox4 en fibroblastos del corazón (Ellmark et al. 2005), pulmón (Sturrock et al. 2007), en células del músculo liso de la arteria pulmonar (Sturrock et al. 2006) y en hepatocitos (Carmona-Cuenca et al. 2006). En respuesta a TGF-β, la expresión de Nox4 tiene diferentes funciones: según el tipo celular promueve proliferación (Sturrock et al. 2006) o regula la progresión del ciclo celular (Sturrock et al. 2007); en células del músculo liso de la arteria pulmonar; puede promover la diferenciación irreversible de fibroblastos cardíacos hacia miofibroblastos (Cucoranu et al. 2005).

Además se ha descrito que Nox4 puede tener un papel importante en diferentes enfermedades crónicas: en asma (Hoidal et al. 2003), en hipertensión pulmonar (Djordjevic et al. 2005; Sturrock et

al. 2006), en arterosclerosis e hipertensión (Brandes 2003; Brandes and Schroder 2008; Frey et al. 2008), o diabetes (Etoh et al. 2003; Block et al. 2009). Al igual que otros miembros de la familia, se ha observado una fuerte correlación entre la expresión de Nox, ROS y células cancerígenas y/o proliferativas, de manera que no es sorprendente encontrar Nox4 expresado en diferentes tipos de tumores: en melanomas (Yamaura et al. 2009), cáncer pancreático (Vaquero et al. 2004; Lee et al. 2007a), glioblastoma (Shono et al. 2008), cáncer de próstata (Tam et al. 2007), o cáncer de tiroides (Weyemi et al. 2009). Además, se ha observado que Nox4 promueve angiogénesis (Datla et al. 2007) y procesos fibróticos tanto en el corazón (Cucoranu et al. 2005; Spurney et al. 2008), como en riñón (Block et al. 2008) y en pulmón (Hecker et al. 2009). Por último destacar que también se ha correlacionado la expresión de Nox4 con enfermedades hepáticas, ya que HCV induce la expresión de Nox4, contribuyendo a la producción de ROS, lo que podría tener relevancia en el daño hepático (Boudreau et al. 2009). Además, después de un proceso de isquemia/reperfusión en el hígado hay una activación de Nox2 y Nox4 que promueven la proliferación celular y, a la vez, la activación de caspasas que median procesos apoptóticos (Marden et al. 2008).

Transición epitelio-mesénquima

Los **procesos de transición epitelio-mesénquima (EMT)** se caracterizan por la inhibición del contacto célula-célula, la reorganización del citoesqueleto, la pérdida de la organización polarizada y la adquisición de un fenotipo invasivo y migratorio (Thiery 2003; Acloque et al. 2009). Durante el proceso de EMT, sobre todo durante el desarrollo y en procesos tumorogénicos, las células pueden conservar características epiteliales y a la vez presentar un gran número de características mesenquimales (Kalluri and Weinberg 2009). Los procesos de EMT se han clasificado recientemente en tres tipos según su contexto biológico y los biomarcadores expresados: Tipo I, cuando ocurren durante diferentes fases de la embriogénesis; Tipo II, que se inicia como un proceso de reparación y/o fibrosis tisular; y tipo III, que es el resultado de cambios genéticos y epigenéticos en células cancerígenas que promueven la invasión y propagación de las células tumorales (Kalluri 2009). El proceso es reversible. Así, la transición mesénquima-epitelio (MET) tiene lugar en cualquiera de los tipos de EMT: en el tipo I para dar lugar a tejidos epiteliales secundarios, en el II durante el proceso de "curación" de un tejido fibrótico, y en el tipo III para permitir la formación de nódulos metastásicos tras la diseminación de las células tumorales (Hollier et al. 2009; Kalluri and Weinberg 2009).

Varios estímulos pueden inducir un proceso de EMT, por ejemplo Hedgehog, EGF, HGF, miembros de la familia TGF-β, Wnt, FGF y miembros de la familia IGF (Barrallo-Gimeno and Nieto 2005). Todos ellos, a su vez, lo hacen a través de diferentes factores de transcripción capaces de dirigir el proceso de EMT: proteínas de la familia de Snail (Snail, Slug) (Nieto 2002), proteínas bHLH (E12/E47, Twist) (Ansieau et al. 2008), o las proteínas ZEB1 y -2 (Eger et al. 2005). Estos factores reprimen la expresión de la caderina-E e incrementan la expresión de la caderina-N típicamente expresada en células mesenquimales, siendo este cambio en la expresión de caderinas una de las principales características de la EMT (Zeisberg and Neilson 2009). Además de regular la expresión de caderina E, estos factores de transcripción inhiben la expresión de otros genes implicados en mantener la estructura y función de las células epiteliales, por ejemplo claudinas y ocludinas (Vandewalle et al. 2005; Ansieau et al. 2008; Moreno-Bueno et al. 2008), o citoqueratinas (CK17, 18, 19 y 20) (Ikenouchi et al. 2003; De Craene et al. 2005). Además, inducen la expresión de proteínas mesenquimales como fibronectina, vitronectina, caderina-N, y vimentina (Vandewalle et al. 2005; Bindels et al. 2006; Ansieau et al. 2008; Xu et al. 2009), y de proteínas implicadas en la migración e invasión, como metaloproteasas (MMPs) (Jorda et al. 2005; Miyoshi et al. 2005; Taki et al. 2006) y

RhoB (Xu et al. 2009). Otros mecanismos moleculares que juegan un papel relevante en la inducción de un proceso de EMT son: la translocación nuclear de β-catenina (Nelson and Nusse 2004), la vía de ephrinB1 (Lee et al. 2008), o Notch (Wang et al. 2009b) y la señalización vía integrinas (Li et al. 2003; Qian et al. 2005; White et al. 2007). Recientemente se ha descrito que la expresión de microRNAs específicos regula EMT mediada por TGF-β (Zavadil et al. 2007; Gregory et al. 2008), o Twist (Ma et al. 2007a).

La inducción de un proceso de EMT no sólo resulta en la adquisición de características mesenquimales, sino que también puede tener como consecuencia la adquisición de características de célula madre y la adquisición de resistencia a apoptosis. El tratamiento crónico de hepatocitos fetales con TGF-β permite seleccionar una población celular que ha sufrido un proceso de EMT, es resistente a apoptosis y muestra características de células progenitoras, bipotenciales, del hígado (Valdes et al. 2002; Del Castillo et al. 2006; del Castillo et al. 2008). La inducción de EMT, ya sea por sobreexpresión de Twist, Snail o RAS, o por tratamiento con TGF-β, es coincidente con la aparición de una población con características de célula madre ya que son CD44+/CD24- y pueden formar mamosferas (Mani et al. 2008). Se ha visto que la expresión de Wnt es necesaria para mantener un estadio de des-diferenciación y la capacidad de auto-renovación en células metastáticas, mediante la expresión de Slug y Twist (DiMeo et al. 2009). Por otro lado, Snail inhibe proliferación celular, ya que reprime la expresión de ciclina D (Vega et al. 2004), y protege de la muerte inducida por señales de estrés, daño al DNA o factores pro-apoptóticos como el TGF-β (Kajita et al. 2004; Martinez-Alvarez et al. 2004; Vega et al. 2004). Además, se ha observado que la sobreexpresión de inductores de EMT, como Snail o Slug, promueve supervivencia celular (Vega et al. 2004) y la adquisición de quimiorresistencia en células tumorales (Kurrey et al. 2009). La sobreexpresión de Twist también conduce a la adquisición de resistencia a cisplatino o paclitaxel, entre otros (Hollier et al. 2009).

El factor de crecimiento transformante tipo beta (TGF-β)

La familia del **TGF-β** está formada por 33 miembros en humanos. Entre ellos encontramos las proteínas morfogenéticas del hueso (BMPs), la activina/inhibina, los factores de crecimiento y diferenciación (GDF)s, nodal, y hormonas anti-Müllerian. Los miembros de esta familia regulan numerosos procesos celulares que incluyen proliferación y diferenciación celulares, apoptosis, producción de matriz extracelular, o migración, de manera que están implicadas en el desarrollo y en procesos fisiológicos normales, pero también en circunstancias patológicas, como cáncer, fibrosis y enfermedades autoinmunes (Pardali and Moustakas 2007; Ross and Hill 2008).

El TGF-β es secretado como un polipéptido inactivo que necesita ser procesado mediante proteolización. El ligando procesado puede unirse directamente al receptor tipo II de TGF-β (TβRII) o bien a receptores tipo III (β-glycan, endoglin) que facilitan su unión al TβRII (Pardali and Moustakas 2007). Una vez el ligando se ha unido al TβRII, que está constitutivamente activo, éste heterodimeriza con el TβRI, fosforilándolo y activándolo. A su vez, el TβRI fosforila a las proteínas Smads receptoras (Smad2 y 3 en el caso de señalización por TGF-β), que forman un complejo con la co-Smad, Smad4, que es translocado al núcleo donde interacciona con otros factores de transcripción, induciendo o inhibiendo la expresión de varios genes (Ross and Hill 2008; Heldin et al. 2009). En el núcleo, las R-Smads pueden ser defosforiladas por fosfatasas nucleares, de manera que pueden ser exportadas al citoplasma donde podrán volver a interactuar con el TβRI. La activación de las R-Smads puede ser facilitada por varias proteínas (Siegel et al. 2003a; Heldin et al. 2009; Kang et al. 2009). Igualmente, la fosforilación de las R-Smads puede ser impedida por las Smads inhibidoras, I-Smads. En el núcleo las R-Smads se unen a los SBE (Smad Binding Elements), aunque su afinidad al

DNA es baja, y por ello necesitan interactuar con otros factores de unión al DNA (Siegel et al. 2003a; Ross and Hill 2008). Se unen tanto a factores de transcripción con actividad HAT (acetilasas de histonas), promoviendo la unión de factores de transcripción al DNA; como a factores de transcripción con actividad HDAC (deacetilasas de histonas) que previenen la unión al DNA, reprimiendo de esta manera la expresión de algunos de sus genes diana (Ross and Hill 2008). Algunos factores co-represores atenúan la acción de las Smads (Siegel et al. 2003a), ya sea por interacción directa con las Smads, o por unirse al DNA en el mismo sitio de unión que las Smads.

Además de la activación de las Smads, que conduce a cambios en la expresión de genes específicos, el TGF-β también puede inducir otras vías comunes a otros factores extracelulares, incluyendo MEK/ERKs, PI3K, PP2A, Rho, que pueden regular diferentes respuestas biológicas inducidas por el TGF-β (Pardali and Moustakas 2007; Yu et al. 2008). En el caso de activación de la vía PI3K/AKT o MEK/ERKs, el TGF-β lo puede hacer transactivando el EGFR (Vinals and Pouyssegur 2001; Murillo et al. 2005).

El TGF-β induce inhibición del crecimiento y parada del ciclo celular en fase G1 regulando la expresión de varios genes críticos para la progresión del ciclo celular: 1) induce la expresión de p15INK4b y p21CIP1 (Hannon and Beach 1994; Polyak et al. 1994; Datto et al. 1995) que se unen a CDK4 y 6 inhibiendo su actividad; 2) reprime la expresión de CDK4 (Ewen et al. 1995); 3) suprime la expresión de Cdc25A, responsable de activar varias CDKs (Iavarone and Massague 1997; Nagahara et al. 1999); 4) disminuye la expresión de ID1-3 (Siegel et al. 2003b; Kowanetz et al. 2004) y de c-myc (Chen et al. 2002), factores de transcripción que potencian la proliferación. En hepatocitos, el TGF-β induce parada del ciclo celular (Sanchez et al. 1996), y contrarresta las señales proliferativas inducidas por EGF o Insulina (Carr et al. 1986; Sanchez et al. 1998).

El TGF-β es un inductor de apoptosis, ya sea mediante la regulación transcripcional de varios genes implicados en la maquinaria apoptótica o a través de la activación de JNK/p38 u otras vías de señalización (Pardali and Moustakas 2007). El TGF-β puede inducir apoptosis a través de la vía extrínseca de una manera independiente de Fas, ya que activa a la caspasa-8 y la proteolisis de BID (Kim et al. 2004a). Se ha descrito que la proteína adaptadora Daxx (Death Domain Associated Protein) es necesaria para la muerte inducida por TGF-β, a través de su unión al TβRII y activando JNK (Perlman et al. 2001; Padua and Massague 2009). La activación de p38MAPK es esencial en la muerte inducida por TGF-β en células epiteliales de mama (Yu et al. 2002) y en células B (Schrantz et al. 2001), pero no en hepatocitos fetales (Herrera et al. 2001c). Además, diferentes estudios han descrito que el TGF-β induce la producción de ROS coincidiendo con un proceso de apoptosis en células epiteliales, como los hepatocitos fetales de rata (Sanchez et al. 1996), o en fibroblastos de pulmón (Thannickal and Fanburg 1995), un proceso que requiere la síntesis *de novo* de proteínas (Sanchez et al. 1997). Dos mecanismos diferentes podrían estar implicados en la producción temprana de ROS: en primer lugar, un sistema NADPH oxidasa inducible es responsable del aumento inicial de ROS extra mitocondriales (Herrera et al. 2004); en segundo lugar, el TGF-β podría aumentar los niveles de ROS al disminuir la expresión de proteínas antioxidantes, como la catalasa, MnSOD o γ-glutamylcisteinil sintetasa (Franklin et al. 2003; Herrera et al. 2004) y glutatión sintetasa, transferasa, reductasa y peroxidasa (Coyle et al. 2003). El incremento de los niveles de ROS inducido por TGF-β es necesario para el proceso apoptótico en hepatocitos (Sanchez et al. 1996), fibroblastos (Langer et al. 1996) o en células epiteliales de la lente humana (Yao et al. 2007). Las ROS son necesarias para una ejecución eficaz de la apoptosis mitocondrial (Herrera et al. 2001a; Herrera et al. 2001b).

Por último, el TGF-β puede modular la expresión de diferentes miembros de la familia BCL-2. Respecto a los miembros anti-apoptóticos de la familia BCL-2, algunos artículos muestran que el TGF-β disminuye la expresión de BCL-2 (Francis et al. 2000) y BCL-XL (Herrera et al. 2001b; Spender et al. 2009). Sin embargo, otros autores han proporcionado información que muestra que el TGF-β incrementa la expresión de BLC-XL (Prehn et al. 1996; Valdes et al. 2004), o MCL1 (Gingery et al. 2008). El TGF-β también induce la expresión de las proteínas pro-apoptóticas BMF y BIM, promoviendo la vía intrínseca de apoptosis (Ramjaun et al. 2007; Yu et al. 2008) y también se ha demostrado que esta citoquina promueve la expresión de BAX (Teramoto et al. 1998) y BIK (Spender et al. 2009). Además, el TGF-β puede disminuir la expresión de algunos miembros de la familia IAP como survivina (Yang et al. 2008a), BIRC3/cIAP2/HIAP1 (Yu et al. 2008) y XIAP (Wang et al. 2008a); y también promueve su proteolización mediante la acción de las caspasas para disminuir sus niveles proteicos (Herrera et al. 2002). Es interesante señalar que la apoptosis inducida por TGF-β también puede ser mediada por ARTS (Larisch et al. 2000), una proteína mitocondrial de semejanza a septina que se une y disminuye la expresión de XIAP contrarrestando las propiedades anti-apoptóticas de la familia de IAP (Gottfried et al. 2004).

El TGF-β también puede inducir señales anti-apoptóticas a través de la activación de AKT (Valdes et al. 2004; Wilkes et al. 2005; Song et al. 2006), que a su vez pueden prevenir la apoptosis inducida por TGF-β de diferentes formas. Por ejemplo, AKT puede fosforilar a BAD y prevenir su asociación con BCL-XL y también puede impedir la activación transcripcional de Foxo1 (Valverde et al. 2004). Además, AKT puede interactuar con Smad3 y evitar su translocación al núcleo, bloqueando así su capacidad para inducir la expresión de los genes necesarios para la apoptosis inducida por el TGF-β (Conery et al. 2004). Sin embargo, muy a menudo la activación de AKT es transitoria y después del tratamiento con TGF-β los niveles de fosfo-AKT disminuyen (Valdes et al. 2004). Esto podría estar relacionado con el hecho de que en respuesta a TGF-β se regulan también otros genes que favorecen los efectos supresores del TGF-β, tales como SHIP, una fosfatasa de lípidos cuya expresión aumenta en respuesta al TGF-β y que bloquea la fosforilación de fosfolípidos mediada por PI3K y, por lo tanto, inhibe la vía de AKT (Valderrama-Carvajal et al. 2002). La activación temprana de AKT podría estar relacionada con la capacidad del TGF-β de transactivar las vías de c-SRC y del receptor de EGF (Park et al. 2004; Murillo et al. 2005). De hecho, el TGF-β podría mediar la producción autocrina de ligandos del receptor de EGF, lo que confiere resistencia a sus efectos pro-apoptóticos en hepatocitos (Del Castillo et al. 2006; Murillo et al. 2007). Sin embargo, la capacidad de los hepatocitos para sobrevivir al TGF-β es dependiente del grado de diferenciación celular (Sanchez et al. 1999). Los hepatocitos fetales de rata responden al TGF-β induciendo señales de supervivencia; en cambio, los hepatocitos adultos de rata no (Caja et al. 2007). Estas diferencias podrían ser debidas a los bajos niveles de AKT y a la baja expresión y actividad de TACE/ADAM17 (responsable de la proteolización de los ligandos del receptor de EGF) que se observa en los hepatocitos adultos.

El papel de Smads en la apoptosis inducida por TGF-β aún no está completamente establecido. Sin embargo, diferentes estudios apoyan un papel para Smad3. Así, en hepatocitos Smad3 es necesaria para que el TGF-β induzca parada del ciclo celular y apoptosis (Ju et al. 2006; Yu et al. 2008) y se ha demostrado que la sobreexpresión de Smad3 promueve la actividad pro-apoptótica a través de la señalización de TGF-β y la activación de la vía p38 MAPK, protegiendo al hígado de hepatocarcinogénesis (Yang et al. 2006). Se ha sugerido que en las células FaO de hepatoma de rata el TGF-β induce apoptosis a través de la proteolización de BAD de una manera dependiente de Smad3 (Kim et al. 2002a). Finalmente, en hepatocitos se ha descrito que Smad3 (Black et al. 2007) y Smad4 (Ramjaun et al. 2007) podrían ser necesarias para la apoptosis inducida por el TGF-β. El incremento

en la expresión de BIM y BMF inducido por el TGF-β es dependiente de Smad4, p38 y la generación de ROS (Ramjaun et al. 2007).

Otras de las funciones principales del TGF-β es la inducción de procesos de EMT. El TGF-β vía Smads induce la expresión de varios factores de transcripción: Snail1 y 2, ZEB1 y 2, E47, E2-2 y Twist (Peinado et al. 2004; Peinado et al. 2007). Smad3 es indispensable para que el TGF-β induzca un proceso de EMT, tanto *in vivo* como en cultivo celular (Millet and Zhang 2007). Además, otros efectores independientemente de las Smads también contribuyen al establecimiento de EMT, por ejemplo RAS (Yue et al. 2004), PI3K (Zhang 2009), RhoA (Ozdamar et al. 2005), Notch (Zavadil et al. 2004), ERK1/2, y NF-κB (Pardali and Moustakas 2007). En los últimos años, diferentes estudios han descrito que el TGF-β induce un proceso de EMT en hepatocitos y en las células de cáncer de hígado (Sanchez et al. 1999; Rossmanith and Schulte-Hermann 2001). En este sentido, el TGF-β es conocido por desencadenar un proceso de EMT a través de inducir la expresión de Snail y Slug en condiciones fisiológicas y patológicas (Spagnoli et al. 2000; Gotzmann et al. 2002; Valdes et al. 2002; Sugimachi et al. 2003; Miyoshi et al. 2005). El proceso de EMT inducido por el TGF-β en hepatocitos es acompañado por un proceso de desdiferenciación celular (Sanchez et al. 1999; Valdes et al. 2002). Snail reprime HNF4α, lo que resulta en la pérdida de marcadores epiteliales y la expresión de proteínas mesenquimales (Cicchini et al. 2006). Resultados recientes han sugerido que el TGF-β puede transdiferenciar los hepatocitos fetales de rata hacia células progenitoras hepáticas con capacidad de diferenciarse en hepatocitos maduros y colangiocitos (del Castillo et al. 2008).

Es importante señalar que a menudo señales pro-apoptóticas y señales de inducción de EMT ocurren simultáneamente en respuesta al TGF-β, como en el caso de los hepatocitos fetales de rata. El éxito del proceso de EMT depende inicialmente de la capacidad de las células de evitar la muerte celular inducida por el TGF-β. Sin embargo, más adelante el proceso de EMT media señales de supervivencia que rescatan a las células de las señales de muerte (Valdes et al. 2002). En este sentido, como se ha comentado anteriormente, la sobreexpresión de Snail protege a las células de apoptosis (Vega et al. 2004). El proceso de EMT podría conferir resistencia a los efectos supresores del TGF-β aumentando la expresión de los ligandos del EGFR y activando de la vía de receptor de EGF, lo que de nuevo apunta a la existencia de una interacción entre las señales del TGF-β y las del EGF en hepatocitos (Del Castillo et al. 2006).

El TGF-β actúa como supresor tumoral en los primeros estadios de un proceso tumorogénico, pero en estadios más avanzados esta citoquina contribuye a la progresión tumoral, facilitando procesos de invasión y metástasis, mediante su capacidad de inducir EMT, aumentar la expresión de algunos factores mitogénicos, así como actuar sobre las células del estroma tumoral promoviendo angiogénesis (Pardali and Moustakas 2007; Massague 2008; Heldin et al. 2009). El TGF-β también actúa como supresor del sistema inmune (Siegel and Massague 2003; Pardali and Moustakas 2007). Muchos tumores pierden la respuesta a las señales supresoras del TGF-β debido a mutaciones en las R-Smads, Smad4 o en los receptores (Pardali and Moustakas 2007), o bien debido a que otras vías de señalización, como las vías RAS/Raf/MEK, PKC o EGFR, contrarrestan las señales de la vía del TGF-β (Wakefield and Roberts 2002). En estas condiciones, los efectos pro-tumorogénicos prevalecen y el TGF-β se convierte en una citoquina clave para la progresión e invasividad del tumor.

El factor de crecimiento epidérmico EGF

La **familia de receptores del EGF: ERBB** está compuesta por cuatro receptores ERBB1-4. Sus ligandos extracelulares (EGF, TGF-α, amfiregulina, epigenina, netacelulina, HB-EGF, epiregulina y

las neuregulinas) contienen el domino EGF (epidermal growth factor) (Citri and Yarden 2006). Las vías de señalización activadas por el EGFR regulan la expresión de genes implicados en progresión del ciclo celular, supervivencia, diferenciación, y migración celular. De los cuatro receptores, dos de ellos no son autónomos, ERBB2 y 3, ya que carecen de la capacidad de interactuar con los ligandos o de la actividad quinasa, respectivamente; a pesar de ello sí que forman complejos heterodímericos con el resto de ERBB y son capaces de generar señales intracelulares. Los ligandos unidos a membrana pueden ser activos biológicamente a través de señalización yuxtacrina, aunque en la mayoría de los casos el dominio extracelular es proteolizado por metaloproteasas de la familia ADAM (a disintegrin and metalloprotease) (Berasain et al. 2009), cuyos miembros más relevantes para este proceso son ADAM10 y ADAM17/TACE (Borrell-Pages et al. 2003). La actividad de las ADAMs y en particular de ADAM17 está muy regulada y puede ser inducida por varios estímulos, como Angiotensina II, LPa, endotelina, IL-8, PGE2 (Berasain et al. 2009), TRAIL (Van Schaeybroeck et al. 2008) y TGF-β (Murillo et al. 2005; Wang et al. 2008b; Wang et al. 2009a).

Una vez el ligando se ha unido al receptor se produce la autofosforilación de varios residuos tirosina, creando sitios de unión a varias proteínas como Shc, Grb7, Grb2, Crk, fosfolipasa Cc (PLCc), las kinasas SRC y PI3K, las proteínas fosfatasas SHP1 y SHP2, y la ubiquitin ligasa Cbl E3. Hay otras proteínas que se activan a través de las proteínas adaptadoras como la fosfolipasa D, y STAT1, 3 y 5. Por ejemplo, las proteínas adaptadoras Grb2 y Shc reclutan a RAS y activan la vía de las MAPK (Jorissen et al. 2003). La inactivación de la vía del EGFR se produce a través de desfosforilación o por disminución del receptor a través de su internalización y su consecuente degradación por lisosoma (Zandi et al. 2007), aunque cabe mencionar que cada vez hay más evidencias de que los receptores internalizados son capaces de continuar señalizando, aunque parece que las vías activadas son diferentes a las activadas cuando el receptor se encuentra en la superficie celular (Citri and Yarden 2006).

En varios tipos de tumores se ha descrito la actividad aberrante o incrementada del EGFR, y se ha observado que eso coincide con un peor pronóstico. Actualmente se sabe que la vía del EGFR puede ser desregulada por varios mecanismos: 1) incremento de la producción de ligandos; 2) incremento de los niveles de proteína de EGFR; 3) mutaciones del EGFR que inducen una activación constitutiva del receptor; 4) defectos en la disminución y reciclaje del EGFR; y 5) interacción con otros receptores como el IGFR o c-MET (Zandi et al. 2007; Hynes and MacDonald 2009).

En el hígado adulto se expresan los niveles más elevados de EGFR en comparación al resto de células no transformadas (Dunn and Hubbard 1984). Los ligandos del EGFR tienen una gran capacidad mitogénica (Fausto et al. 1995; Block et al. 1996; Mitchell et al. 2005) y una función anti-apoptótica (Fabregat et al. 1996; Fabregat et al. 2000) en los hepatocitos; además de tener un papel pro-regenerativo (Berasain et al. 2007). La expresión de ADAM17 también es incrementada durante un proceso de regeneración hepática (Lin et al. 2008). Al igual que en otros tipos tumorales, se ha observado un incremento en la expresión de los ligandos del EGFR en HCC (Jorissen et al. 2003).

Antecedentes del grupo

En los últimos años nuestro grupo ha estudiado las diferentes **vías de señalización inducidas por TGF-β en hepatocitos fetales de rata**. A dosis bajas, el TGF-β inhibe el crecimiento, pero a concentraciones más elevadas es capaz de inducir apoptosis (Sanchez et al. 1995; Sanchez et al. 1996). El proceso apoptótico está mediado por un incremento en el contenido intracelular de ROS,

dependiente de la *síntesis de novo* de proteínas, y que correlaciona con una caída en los niveles intracelulares de glutatión (Sanchez et al. 1997). La muerte inducida por TGF-β en estas células se correlaciona con un descenso en los niveles de Bcl-xL, la despolarización de la membrana mitocondrial, la salida de citocromo c y posterior activación de caspasas (Herrera et al. 2001a; Herrera et al. 2001b). El incremento de ROS intracelulares se produce por activación de un sistema NADPH oxidasa y por disminución en la expresión de proteínas antioxidantes (Herrera et al. 2004). Recientemente hemos descrito que la NADPH oxidasa NOX4 se induce en condiciones pro-apoptóticas (Carmona-Cuenca et al. 2006), pero otras NADPH oxidasas podrían jugar un papel diferente en la señalización inducida por TGF-β (Murillo et al., 2007). La muerte inducida por TGF-β puede ser inhibida por EGF a través de la activación de PI3K (Fabregat et al. 2000), que contrarresta la expresión de Nox4 (Carmona-Cuenca et al. 2006).

Sin embargo, el 40-50% de las células sobreviven a los efectos apoptóticos del TGF-β y adquieren una morfología fibroblastoide (Sanchez et al. 1999). Esto es debido a que el TGF-β también induce señales anti-apoptóticas en los hepatocitos fetales, proceso que requiere la activación del EGFR, producida por un aumento en los niveles de expresión de sus ligandos y activación de la metaloproteasa TACE/ADAM17 que los proteoliza y activa (Valdes et al. 2004; Murillo et al. 2005; Del Castillo et al. 2006; Murillo et al. 2007). Las células que sobreviven al TGF-β responden a esta citoquina en términos de migración e invasión, disminuyendo la expresión de marcadores hepáticos (Sanchez et al. 1999), e induciendo un proceso de EMT (Valdes et al. 2002). La población mesenquimática resultante es resistente a la muerte inducida por TGF-β, ha sufrido un proceso de desdiferenciación y expresa marcadores de célula madre (Del Castillo et al. 2006; del Castillo et al. 2008). Esta población puede rediferenciarse tanto a un linaje hepatocítico como hacia células biliares cuando se mantienen con los medios de diferenciación adecuados.

Por último, resultados preliminares al inicio de esta tesis doctoral proponían que la doble respuesta al TGF-β observada en hepatocitos fetales de rata en cultivo primario era exclusiva de este estadio del desarrollo hepático, ya que en hepatocitos adultos de rata el TGF-β sólo inducía apoptosis. La incapacidad del TGF-β de inducir señales de supervivencia parece deberse a la baja expresión de AKT y de TACE observada en hepatocitos adultos. Además, el TGF-β era incapaz de inducir un proceso de EMT en hepatocitos adultos de rata. A la vista de estos resultados se consideró de gran importancia analizar cuál podría ser la respuesta al TGF-β en células tumorales hepáticas.

Así, nuestro principal objetivo en esta tesis ha sido analizar si las células de carcinoma hepatocelular responden a la muerte celular inducida por el TGF-β, y en el caso de que hayan adquirido resistencia, estudiar los mecanismos moleculares que la confieren. También queríamos saber si el TGF-β induce señales de supervivencia y un proceso de EMT en células tumorales hepáticas, y la relevancia de este proceso en la progresión del tumor hepático. Aunque quisimos iniciar el estudio con células de hepatoma de rata, debido a nuestra experiencia en este modelo de celular, consideramos muy importante también analizar la situación en células tumorales de hígado humano, ya que es conocido que los niveles de TGF-β son elevados en carcinoma hepatocelular (HCC) y diferentes evidencias han sugerido que la respuesta al TGF-β está alterada en células de HCC.

OBJETIVOS

Objetivo general: ANALIZAR LAS SEÑALES INTRACELULARES INDUCIDAS POR TGF-β EN CÉLULAS TUMORALES DEL HÍGADO PARA COMPRENDER MEJOR SU PAPEL EN HEPATOCARCINOGÉNESIS

Objetivos específicos:

1. Análisis de la respuesta al TGF-β en células tumorales del hígado en términos de muerte. Papel de Nox4. Posible papel del EGFR en las señales de supervivencia inducidas por TGF-β.

2. Análisis de las señales de supervivencia intracelulares, independientes de la vía del EGFR, que puedan contrarrestar la apoptosis inducida por TGF-β en células de hepatocarcinoma celular humano.

3. Efectos del TGF-β sobre el fenotipo y el estado de diferenciación de las células hepáticas.

Resultados

El TGF-β induce una respuesta dual en las células de hepatoma de rata FaO, activando tanto apoptosis como señales de supervivencia, estas últimas mediadas por la activación del EGFR coincidente con la inducción de la expresión de sus ligandos y su activación por la metaloproteasa TACE/ADAM17 (Figs. 1-3). La activación del EGFR resulta en la fosforilación de AKT y SRC. Al realizar estos experimentos observamos que la vía del EGFR se encuentra basalmente activada en estas células, incluso en ausencia de suero. La inhibición del EGFR con la tirfostina AG1478 bloquea la inducción de señales de supervivencia y potencia la muerte inducida por TGF-β coincidente con un incremento en la expresión de Nox4 (Figs. 1-4).

Estos resultados nos llevaron a estudiar la respuesta al TGF-β en células de hepatocarcinoma celular humano. Para ello, primero quisimos estudiar los efectos de esta citoquina en hepatocitos fetales humanos (HFH), en colaboración con el Dr. Nelson Fausto en el Departamento de Patología, Universidad de Washington. Por resultados previos del grupo sabíamos que los HFH responden al TGF-β induciendo apoptosis. En este trabajo observamos que la inhibición del EGFR, mediante el inhibidor Gefitinib, inhibe el crecimiento de los HFH, pero también potencia los efectos citotóxicos inducidos por TGF-β (Fig. 5), incrementando la expresión de *NOX4* inducida por TGF-β (Fig. 6A). El tratamiento con TGF-β per se induce la expresión de los genes pro-apoptóticos de la familia BCL-2 *BMF* y *BIM*, pero a la vez induce la expresión de los miembros anti-apoptóticos *BCL-XL* e inhibidores de caspasas, como *XIAP*. El tratamiento conjunto con Gefitinib contrarresta la inducción de los genes anti-apoptóticos por TGF-β. Además, la combinación TGF-β + Gefitinib disminuye la expresión de *HIAP1* (Fig. 6B).

El TGF-β induce diferentes respuestas en términos de viabilidad en las cuatro líneas celulares de hepatocarcinoma celular humano analizadas (Hep3B, HepG2, PLC/PRF/5 y SK/Hep1). Dos de estas líneas responden a los efectos de inhibición del crecimiento del TGF-β (Hep3B y PLC/PRF/5), en cambio las otras dos líneas son resistentes (HepG2 y SK-Hep1) (Fig. 7). La diferencia entre ellas es la capacidad de inducir la expresión de *NOX4*, la activación de caspasas y el incremento del porcentaje de células hipodiploides (Figs. 8 y 10). En las células Hep3B observamos que el silenciamiento de la

expresión de NOX4 bloquea la apoptosis inducida por TGF-β (Fig. 11). En dichas células, el TGF-β induce la expresión de los genes pro-apoptóticos de la familia BCL-2 BIM y BMF, pero también la expresión de los miembros anti-apoptóticos BCL-XL y MCL1 (Fig. 9). Adicionalmente, el TGF-β activa otras señales anti-apoptóticas mediante la activación del EGFR, aumentando los niveles de fosforilación de SRC, AKT y ERK1/2 (Fig. 12). De forma semejante a las células de hepatoma de rata FaO, las células Hep3B presentan una activación basal del EGFR.

La inhibición del EGFR mediante experimentos de silenciamiento con siRNA induce la pérdida de viabilidad y la inducción de la actividad caspasa-3 (Fig. 13). Además, potencia los efectos del TGF-β en términos de inhibición de crecimiento y apoptosis, potenciando la inducción de *BIK*, *BIM* y *BMF*, y disminuyendo la expresión de *HIAP1*, a la vez que potencia la inducción de la expresión de *NOX4* (Figs. 14-15). A nivel de viabilidad y apoptosis, observamos que el uso de los inhibidores del EGFR, AG1478 o Gefitinib, también potencia los efectos del TGF-β tanto en las células Hep3B como en las PLC/PRF/5, ambas sensibles a los efectos citotóxicos del TGF-β (Figs. 16-17). Sin embargo, el uso de EGFR siRNA o inhibidores del EGFR no sensibiliza a la muerte inducida por TGF-β en las líneas celulares resistentes a esta citoquina (HepG2 y SK-Hep1) (Figs. 18-19).

Estos resultados dirigieron nuestra atención al estudio de señales de supervivencia intracelulares que podrían ser responsables de conferir resistencia a la muerte por TGF-β en células de HCC, concentrando nuestros experimentos en la línea celular HepG2. Entre diferentes inhibidores de quinasas, observamos que el uso de un inhibidor de MEK (PD98059, U0126 o PD0325901) o el silenciamiento de ERK1/2 por siRNA sensibiliza a estas células a la muerte por TGF-β (Fig. 20, 23 y 24). Estos resultados podrían explicarse por el hecho de que estas células tienen una mutación en N-RAS, y presentan una hiperactivación de las ERKs. La inhibición de MEK también potencia la muerte por TGF-β en Hep3B y PLC/PRF/5 que también presentan una activación basal de las ERKs, aunque menor que la observada en HepG2 (Fig. 21). Al estudiar el mecanismo por el cual la inhibición de MEK (PD98059) potencia la muerte por TGF-β observamos que: 1) no coincide con cambios en la expresión de los efectores de la vía TβRs/Smads (Fig. 22); 2) coincide tanto con la pérdida de potencial de membrana de la mitocondria como con la permeabilización del lisosoma (Fig. 23); 3) el co-tratamiento induce la expresión de BIM y BMF, a la vez que el tratamiento con el inhibidor de MEK disminuye la expresión de BCL-XL y MCL1, efecto que es mantenido cuando se añade el TGF-β (Fig. 24); 4) aumenta la producción de ROS y disminuyen los niveles intracelulares de GSH (Fig. 26); y 5) la inhibición de MEK permite la inducción de NOX4 en respuesta al TGF-β (Fig. 26). Tanto el uso de antioxidantes, GEE y/o DPI, como el silenciamiento de la expresión de NOX4 con siRNA impiden la inducción de apoptosis inducida por TGF-β + PD98059 (Fig. 26, 28 y 30), a la vez que bloquean los cambios en la expresión de los miembros de la familia BCL-2 (Fig. 29-30). Sin embargo, el silenciamiento de NOX4 no recupera la permeabilidad lisosomal (Fig. 28), pero observamos que el uso del inhibidor de Catepsina B (Ca-074) protege ligeramente de la muerte inducida por TGF-β + PD98059 (Fig. 31), de manera que la Catepsina B puede contribuir a la apoptosis inducida por TGF-β + PD98059 en paralelo e independientemente al mecanismo inducido por NOX4.

Finalmente, hemos analizado los efectos del tratamiento con TGF-β sobre el fenotipo celular. En el caso de las células FaO, HFH y Hep3B las células que sobreviven a los efectos apoptóticos adquieren una morfología fibroblastoide (Fig. 32, 35 y 39). En las células FaO, observamos que el TGF-β induce la expresión de *Snai1*, disminuye la expresión de caderina E, promueve la reorganización del citoesqueleto de actina con la aparición de prolongaciones de la membrana celular, lamelipodios y fibras de estrés. Asimismo, hay cambios en la expresión de proteínas de los filamentos intermedios,

con disminución de las específicas de células epiteliales, como CK18 (Fig. 32-33). Mediante tratamiento crónico con TGF-β hemos aislado la población mesenquimal, que denominamos TβT-FaO (de "TGF-β-treated FaO cells). Estas células muestran un claro fenotipo fibroblastoide, han sufrido un proceso de des-diferenciación (pérdida de la expresión de *Hnf1*α y *Hnf4*), y muestran expresión del receptor del factor de células madre (stem cell factor), c-*Kit*. Sin embargo, si el TGF-β se retira del medio de cultivo, las células revierten a un fenotipo epitelial (Fig. 34).

En aquellos HFH que sobreviven a los efectos apoptóticos del TGF-β, esta citoquina induce un proceso de EMT promoviendo la expresión de *SNAI1*, *VIMENTINA*, y *CADERINA-N*, y disminuyendo la expresión de *CADERINA-E* (Fig. 35). El tratamiento con TGF-β promueve el reemplazo de los microfilamentos de CK18 por los de Vimentina (Fig. 35). Aunque los HFH muestran características de hepatoblastos, tanto el tratamiento con TGF-β durante 72 horas, como el tratamiento continuado con esta citoquina, no sólo inducen un proceso de EMT, sino también inducen un proceso de des-diferenciación en estas células. Se observa que el TGF-β disminuye la expresión de marcadores hepáticos *ALBÚMINA* y *HNF4A* a nivel de mRNA, y la expresión proteica de Albúmina, alfa-1-antitripsina (A1AT), Conexina 26 (CX26), o el antígeno hepático Hepar1. También induce la expresión del factor de transcripción HNF3β, normalmente expresado en los primeros estadios de hepatogénesis, e incrementa la expresión de los marcadores de hepatoblastos CK7 y CK19 (Fig. 36). Por último, se observa la expresión del marcador de célula madre hematopoyética CD90/THY1 (Fig. 36). Al igual que en hepatocitos fetales de rata (Valdes et al. 2002), o en células FaO de hepatoma de rata (como se ha indicado anteriormente), se aislaron las células que sobreviven a la muerte inducida por TGF-β y adquieren un fenotipo mesenquimal, tratándolas crónicamente con TGF-β. Estas células presentan niveles muy bajos de expresión de Albúmina, A1AT, CX26, y han perdido casi por completo la expresión de Hepar1 y CK18. En cambio expresan niveles más elevados de los marcadores mesenquimales Vimentina y α-SMA, y expresan el marcador de célula madre hematopoyética CD90/THY1 (Fig. 37). La adquisición de un fenotipo mesenquimal y des-diferenciado puede ser revertido al mantener las células con un medio de diferenciación, que les permite recuperar la morfología epitelial, la expresión de marcadores hepáticos *ALBÚMINA*, *HNF4A*, CK18 y CX26, coincidente con disminución en la expresión de *SNAI1* y Vimentina (Fig. 38).

En las células Hep3B el TGF-β también induce un proceso de EMT, ya que induce la expresión de *SNAI1, CADERINA-N,* y *VIMENTINA* y la disminución de la expresión de *CADERINA-E.* Estos cambios son coincidentes con reorganización del citoesqueleto de actina, (Fig. 39). Al igual que en células FaO y HFH, el proceso de EMT inducido por TGF-β es acompañado por un proceso de des-diferenciación (disminución de la expresión de *ALBÚMINA* y *AFP*) y el incremento de marcadores de célula madre (*THY*, *KIT* y *EPCAM*) (Fig. 40).

Discusión

El TGF-β tiene efectos contradictorios durante el desarrollo del hígado y la hepatocarcinogénesis. Por un lado, el TGF-β inhibe la proliferación, evita la transformación celular e induce apoptosis en hepatocitos, y la alteración de la vía de señalización de esta citoquina puede desregular los procesos apoptóticos en HCC (Bissell et al. 2001; Siegel and Massague 2003). Por otro lado, el TGF-β actúa como promotor tumoral induciendo procesos de EMT que aumentan la capacidad migratoria de los hepatocitos (Gotzmann et al. 2002; Valdes et al. 2002). Se ha observado que la pérdida de las señales supresoras de tumores inducidas por TGF-β es un prerrequisito para la progresión de HCC (Yang et

al. 2006), mientras que las células tumorales mantienen las señales pro-tumorogénicas en respuesta a este factor (Wakefield and Roberts 2002; Seoane 2006). Por ello, un mejor conocimiento de las señales mediadas por el TGF-β en hepatocitos humanos y en células hepáticas tumorales, y una mejor compresión de la forma en que otras señales intracelulares pueden afectar a sus efectos supresores, es de gran importancia para el diseño de nuevas terapias que favorezcan los efectos supresores de esta citoquina.

Nuestro grupo había descrito anteriormente que el TGF-β induce apoptosis en hepatocitos fetales y adultos de rata (Sanchez et al. 1996; Caja et al. 2007). En este trabajo, observamos que el TGF-β induce apoptosis en HFH, y en algunas de las células hepáticas tumorales estudiadas, como las células de hepatoma de rata FaO y las líneas celulares humanas Hep3B y PLC/PRF/5. En cambio, otras dos líneas celulares humanas de HCC, HepG2 y SK-Hep1, son resistentes a sus efectos citotóxicos. Varios grupos han descrito anteriormente que miembros de la familia BCL-2 están implicados en la muerte inducida por TGF-β (Chatzaki et al. 2003; Ramjaun et al. 2007; Gingery et al. 2008; Spender et al. 2009). En HFH y en células Hep3B el TGF-β induce la expresión de los miembros pro-apoptóticos de la familia BCL-2, BMF y BIM, pero también induce la expresión de miembros anti-apoptóticos de las familias BCL-2 e IAP, en particular, BCL-XL, MCL1, XIAP (Figs. 6B y 9B). BMF actuaría como *desrepresor* y BIM como *activador* para promover la activación de BAX y BAK, a la vez que secuestrando a las proteínas anti-apoptóticas BCL-XL y MCL1 (Letai et al. 2002; Willis and Adams 2005; Merino et al. 2009). En resumen, el TGF-β induce señales pro- y anti-apoptóticas en hepatocitos fetales humanos y en células tumorales del hígado, y del balance de las mismas dependerá el destino de la célula.

La apoptosis inducida por el TGF-β requiere de la producción de ROS en hepatocitos, proceso que es dependiente de Smad3 (Sanchez et al. 1996; Franklin et al. 2003; Black et al. 2007). En hepatocitos fetales de rata sabíamos que el TGF-β inducía un incremento de los niveles de ROS a través de un sistema NAPDH oxidasa y disminución de los niveles de enzimas antioxidantes (Herrera et al. 2004). En este trabajo hemos observado que la inducción eficiente de apoptosis en respuesta al TGF-β en células hepáticas depende de su capacidad de responder al TGF-β induciendo la expresión de NOX4 (Fig. 6, 8-11). La idea de que los ROS actúan como intermediarios en la señalización celular ha quedado evidenciada tras el descubrimiento de la familia NOX (Bedard and Krause 2007; Brown and Griendling 2009). Se ha propuesto que la enzima NOX4 puede tener efectos opuestos en diferentes tipos celulares según el estímulo que induzca su actividad, de manera que puede estar involucrada en la inducción de apoptosis (McKallip et al. 2006; Palozza et al. 2007; Basuroy et al. 2009; Song et al. 2009), o bien en proliferación y supervivencia (Vaquero et al. 2004; Edderkaoui et al. 2005; Sturrock et al. 2006; Li et al. 2008; Shono et al. 2008; Naughton et al. 2009). Evidencias anteriores habían sugerido que la inducción de NOX4 por TGF-β podría participar tanto en diferenciación (Cucoranu et al. 2005) como en proliferación (Sturrock et al. 2006) celular. En este manuscrito demostramos que la expresión de NOX4 es necesaria para la muerte inducida por TGF-β en hepatocitos humanos y que la ausencia de respuesta al TGF-β en términos de inducción de NOX4 confiere resistencia a sus efectos apoptóticos en células de HCC.

El TGF-β no sólo induce señales pro-apoptóticas, sino que también induce señales de supervivencia. Hemos observado que siempre sobreviven un 50-60% de células a los efectos citotóxicos de este factor. Trabajos anteriores han indicado que el TGF-β promueve señales de supervivencia a través de la activación del EGFR (Vinals and Pouyssegur 2001; Murillo et al. 2005; Wang et al. 2008b), hecho que confirmamos en el presente trabajo donde observamos que el TGF-β induce la activación de AKT, SRC y ERK a través del EGFR (Figs. 2, 3 y 12).

El hepatocarcinoma celular humano es la tercera causa de muerte por cáncer en el mundo. El mejor tratamiento es resección y trasplante, sin embargo, la mayoría de pacientes a los cuales se les ha realizado una resección del hígado presenta recurrencia o metástasis en los primeros 5 años (Aravalli et al. 2008). Por ello, es necesario entender mejor los mecanismos moleculares alterados en carcinogénesis hepática que participan en la iniciación y progresión del tumor y que producen una desregulación en el balance entre señales de muerte y de supervivencia celular (Mott and Gores 2007; Fabregat 2009). Entre las señales de supervivencia desreguladas en HCC se ha propuesto la vía del EGFR (Berasain et al. 2007; Fabregat et al. 2007), que puede encontrarse sobre-activada debido a un aumento en la expresión de sus ligandos (Inui et al. 1994; Chung et al. 2000; Berasain et al. 2005) o a una sobre-expresión del propio EGFR y ERBB3 (Berasain et al. 2009). Nuestros resultados indican que las células tumorales hepáticas tienen una activación basal del EGFR en ausencia de suero (Figs. 2, 12-13), que media proliferación y supervivencia celular, ya que su inhibición atenúa el crecimiento autocrino e induce apoptosis (Figs. 13, 15, 17-19).

Es conocido que la vía de señalización del EGF evita la muerte inducida por TGF-β en hepatocitos y células de hepatoma (Shima et al. 1999; Herrera et al. 2001a; Herrera et al. 2002). Nuestros resultados muestran que la inhibición del EGFR potencia la muerte inducida por TGF-β específicamente en las células que son sensibles a esta citoquina en términos de apoptosis, las células FaO, HFH, PLC/PRF/5 y Hep3B (Figs. 1, 5-6, y 14-17). Sin embargo, la inhibición del EGFR no sensibiliza al TGF-β a las líneas celulares resistentes a esta citoquina (HepG2 y SK-Hep1, Figs. 18-19). Tanto en HFH como en las células de HCC en las cuales la inhibición del EGFR potencia los efectos supresores de tumores del TGF-β, este efecto es coincidente con una mayor inducción de la expresión de NOX4 y una regulación de la expresión de los genes de las familias BCL2 y IAP: 1) Aumentando la inducción mediada por TGF-β de los genes pro-apoptóticos, *BIK*, *BMF* y *BIM*; 2) potenciando la disminución en la expresión de *HIAP1* (Fig. 15); y 3) contrarrestando el incremento inducido por el TGF-β en la expresión de proteínas anti-apoptóticas de ambas familias, BCL-2 y IAP, (Figs. 6, 9 y 15). Otros grupos han descrito que los inhibidores del EGFR promueven apoptosis modulando la expresión génica de la familia BCL-2 (Takaoka et al. 2007; Ling et al. 2008). En HCC se ha observado un incremento de la expresión génica de miembros de la familia IAP (Notarbartolo et al. 2004; Augello et al. 2009) o BCL-2 (Takehara et al. 2001; Fabregat 2009). Por ello, el uso de agentes terapéuticos que directa o indirectamente disminuyan la expresión de estas proteínas anti-apoptóticas incrementará los efectos anti-tumorogénicos del TGF-β. En HCC, la inhibición del EGFR podría ser una buena diana terapéutica ya que inhibiría el crecimiento autocrino de las células de hepatoma, y restauraría la señalización pro-apoptótica del TGF-β, cuyos niveles son elevados en HCC (Song et al. 2002; Dong et al. 2008). Sin embargo, este efecto sólo se daría en células que no presentan sobre-activación en otros mecanismos de supervivencia que no dependan del EGFR.

En HCC se han observado alteraciones en la vía de señalización de RAS (Tarn et al. 2001; Calvisi et al. 2006; Calvisi et al. 2008) y su incremento está asociado a una recurrencia más temprana (Newell et al. 2009). Las alteraciones en la vía de RAS confieren propiedades proliferativas y anti-apoptóticas en células de hígado neoplásicas (Calvisi et al. 2008). En este trabajo hemos observado que la inhibición de la vía MEK/ERK usando inhibidores específicos (PD98059, U0126 o PD325901) (Fig. 23), o el silenciamiento de ERK1/2 (Fig. 24), restaura la respuesta apoptótica del TGF-β en las células resistentes HepG2, y potencia sus efectos citotóxicos en líneas celulares sensibles Hep3B y PLC/PRF/5 (Fig. 21). De esta manera, la sobre-activación de la vía RAS/MEK/ERK en células tumorales hepáticas podría conferir resistencia a los efectos apoptóticos del TGF-β, cuyos niveles de expresión son elevados durante la progresión de HCC (Luo et al. 2006). Se ha descrito que la alteración de los efectos supresores del TGF-β ocurre en estadios avanzados de hepatocarcinogenesis

(Kawate et al. 1999; Yakicier et al. 1999; Coulouarn et al. 2008). Aunque se han descrito alteraciones a nivel de los receptores del TGF-β y SMADS (Yang et al. 2006; Tang et al. 2008), éstas son muy poco frecuentes y otros mecanismos, aun no comprendidos del todo, deben participar en la resistencia a la supresión tumoral. En el caso de la línea celular HepG2, que presenta alteraciones en la vía de RAS (Hsu et al. 1993), observamos que la ausencia de respuesta a los efectos citotóxicos del TGF-β no correlaciona con alteraciones en la vía canónica del TGF-β (Figs. 20 y 22). Proponemos que el punto de conexión entre las vías de señalización de MEK/ERK y TGF-β se podría situar en la NADPH oxidasa NOX4. El TGF-β es incapaz de inducir la expresión de NOX4, la producción de ROS y estrés oxidativo en las células HepG2 (Fig. 26), ya que presentan una fosforilación constitutiva de las ERKs. Nuestros resultados sugieren que la sobre-activación de señales de supervivencia en células de HCC podrían contrarrestar la muerte inducida por TGF-β atenuando el incremento de la expresión de NOX4. En este trabajo describimos por primera vez que la expresión de NOX4 es necesaria para la expresión de las proteínas pro-apoptóticas BMF y BIM (Figs. 25 y 29), cuya expresión ya se había descrito que era ROS dependiente en respuesta al TGF-β (Ramjaun et al. 2007) y aquí demostramos que el sistema productor de ROS es NOX4. En células HepG2, igual que en hepatocitos fetales de rata (Murillo et al. 2005) y células de hepatoma (células FaO y Hep3B), el TGF-β induce la expresión de genes anti-apoptóticos como BCL-XL y MCL1 (Fig. 25). Después del tratamiento con TGF-β y el inhibidor de MEK/ERK se observa una disminución en su expresión, para lo cual la expresión de NOX4 es necesaria (Figs. 25 y 29). Los cambios en la pauta de expresión génica de la familia BCL2 inducidos por el co-tratamiento con TGF-β + inhibidor de MEK resulta en la inducción de apoptosis vía mitocondrial (Figs. 25, 28 y 29), siendo NOX4 y la producción de ROS necesaria para todos estos acontecimientos (Figs. 28-30).

La apoptosis no es sólo mediada por la mitocondria, sino que otros orgánulos, como el lisosoma o el retículo endoplasmático, pueden mediar este proceso. Debido a la localización intracelular de NOX4 nos planteamos si podría estar afectando mecanismos de muerte celular promovidos por otros orgánulos como el lisosoma. La permeabilización lisosomal es una característica clave de la muerte celular inducida por la lipotoxicidad hepática (Anan et al. 2006) y se ha descrito su participación en la muerte en respuesta a TNF-α (Werneburg et al. 2002). Observamos que el co-tratamiento con TGF-β y el inhibidor de MEK induce la permeabilización lisosomal (Fig. 23), mediado por la catepsina B (Fig. 31), pero de una manera independiente de NOX4 (Fig. 28).

En resumen, la sobre-activación de la vía MAPK/ERK en células tumorales de hígado podría jugar un papel en la iniciación y desarrollo de HCC confiriendo resistencia a la muerte inducida por el regulador fisiológico TGF-β. La inhibición de la vía MEK/ERK cambia la respuesta al TGF-β evitando la señalización pro-tumoral y recuperando la señalización supresora de tumores. La molécula clave en este proceso es NOX4, cuya actividad es necesaria para la eficiente regulación de la expresión de proteínas de la familia BCL-2, que controlan la cascada de acontecimientos de la apoptosis mitocondrial.

El TGF-β promueve procesos de EMT mediante mecanismos dependientes e independientes de las Smads (Padua and Massague 2009). En este trabajo se muestra que el TGF-β induce un proceso de EMT en células FaO de hepatoma de rata (Figs. 32 y 33), en hepatocitos fetales humanos (Fig. 35), y en células de hepatocarcinoma humano Hep3B (Fig. 39). Estos resultados indicarían que la EMT inducida por TGF-β podría tener gran importancia durante el proceso de desarrollo del hígado humano, así como en hepatocarcinogénesis.

Resultados anteriores de nuestro grupo habían indicado que el TGF-β induce un proceso de des-diferenciación en hepatocitos fetales de rata (Sanchez et al. 1999; Valdes et al. 2002; del Castillo et al. 2008). Aquí observamos que el proceso de EMT inducido por TGF-β en HFH y en células tumorales del hígado es coincidente con un proceso de des-diferenciación, evidenciado por la disminución de la expresión de factores de transcripción hepáticos expresados en el hepatocito diferenciado (HNF4α o HNF1α), la disminución de marcadores hepáticos (Albúmina, Hepar1, A1AT) y el incremento en la expresión de factores de transcripción característicos de los primeros estadios embrionarios, HNF3β (Figs. 34, 36, 37 y 40). En los diferentes modelos estudiados observamos, asimismo, un incremento en la expresión de las proteínas CK7, Thy1, c-Kit y/o EpCam, proteínas que diferentes grupos han descrito como marcadores de célula madre hepática (Libbrecht and Roskams 2002; Dan et al. 2006; Li et al. 2006b; Schmelzer et al. 2007; del Castillo et al. 2008; Yang et al. 2008d), indicando que el proceso de EMT inducido por el TGF-β es coincidente con un incremento de marcadores de células madre. Además, las células transdiferenciadas obtenidas después del tratamiento con TGF-β presentan rasgos mixtos, mesenquimales y epiteliales, coincidente con la caracterización que otros grupos han hecho de las células madre hepáticas (Dan et al. 2006). Este proceso de trans-diferenciación inducido por el TGF-β es reversible en el caso de los hepatocitos fetales humanos cuando se cultivan en presencia de un medio rico de diferenciación (Fig. 38). Este resultado tendría gran relevancia fisiológica y patológica: por un lado, esto indicaría que después de sufrir EMT, las células humanas podrían repoblar y re-diferenciarse en hepatocitos maduros, lo cual sería de gran interés para el trasplante hepático; por otro lado, en hepatocarcinogenesis el proceso MET podría mediar la formación de nódulos metastásicos (Polyak and Weinberg 2009). En sintonía con los resultados observados en el presente trabajo, se ha sugerido que la inducción de EMT origina un incremento en la población de células madre en células epiteliales mamarias (Mani et al. 2008; Morel et al. 2008).

La pérdida del fenotipo diferenciado, coincidente con la adquisición de propiedades de célula mesenquimal y de célula madre, en células de hígado humano podría sugerir que las células madre tumorales en HCC podrían originarse por des-diferenciación de las células hepáticas normales o pre-neoplásicas. Finalmente, la morfología y características de las células TβT-HFH recuerdan también al fenotipo de los miofibroblastos (Dudas et al. 2007), células que tienen un papel central en fibrosis hepática (Bataller and Brenner 2005). Durante años se creía que los miofibroblastos se originaban a partir de células estrelladas (Kalluri and Neilson 2003), pero cada vez existen más evidencias experimentales que indican que se pueden generar a través de procesos de EMT de células epiteliales normales o cancerígenas, hepatocitos o células biliares (Radisky et al. 2007; Zeisberg et al. 2007c; Dooley et al. 2008; Nitta et al. 2008).

En conjunto, este último bloque de resultados indicaría que la exposición crónica de células hepáticas al TGF-β modula la respuesta a esta citoquina, favoreciendo la selección de células que resisten a sus efectos supresores y desarrollan EMT, proceso que promueve la adquisición de un fenotipo migratorio y desdiferenciado. De alguna manera, este sistema experimental podría mimetizar lo que sucede durante la progresión tumoral en el hígado.

Conclusiones

Primera. Las células hepáticas sensibles a la muerte inducida por el TGF-β responden a esta citoquina induciendo la expresión de la NADPH oxidasa NOX4, que media la producción de especies reactivas de oxígeno (ROS). La inhibición de estos procesos impide que el TGF-β induzca la muerte celular. La expresión de NOX4 es necesaria para una apoptosis mitocondrial eficiente.

Segunda. El TGF-β no sólo induce apoptosis en hepatocitos fetales humanos, o en células de hepatoma, sino que también promueve señales de supervivencia a través de la activación de la vía del EGFR. La activación del EGFR por TGF-β contrarresta sus efectos apoptóticos a dos niveles diferentes: por un lado, la activación del EGFR atenúa el incremento de la expresión de NOX4 y de algunos miembros pro-apoptóticos de la familia BCL-2, como BMF, BIM o BIK; por otro lado, la vía de señalización del EGFR potencia la expresión de proteínas anti-apoptóticas como BCL-XL, MCL1 y XIAP o HIAP, dependiendo del tipo celular.

Tercera. El silenciamiento de la vía del EGFR, o la inhibición de su actividad quinasa, potencian la apoptosis inducida por TGF-β específicamente en células de hepatoma que muestran cierta sensibilidad a la muerte inducida por TGF-β. Sin embargo, algunas líneas celulares, como las células HepG2 o SK-Hep1, que son totalmente resistentes a la citotoxicidad inducida por el TGF-β y no incrementan la expresión de NOX4, no se sensibilizan con inhibidores del EGFR, indicando que otras vías de supervivencia, que no dependen del EGFR, podrían estar evitando las señales del TGF-β.

Cuarta. La sobre-activación de la vía RAS/MEK/ERK evita la muerte inducida por el TGF-β en células de hepatoma. La inhibición de MEK sensibiliza a estas células a responder al TGF-β induciendo la expresión de NOX4 e incrementando la producción de ROS.

Quinta. El silenciamiento de NOX4 con siRNA específico ha demostrado que la activación de esta NADPH oxidasa es necesaria para el incremento de la expresión de BMF y BIM en respuesta al tratamiento combinado de TGF-β y el inhibidor de MEK, a la vez que evita que el TGF-β induzca la expresión de BCL-XL y MCL1.

Sexta. Los hepatocitos humanos no transformados y las células tumorales hepáticas son susceptibles de responder al TGF-β induciendo un proceso de EMT reversible, que es coincidente con la pérdida del fenotipo diferenciado y la adquisición de marcadores de células madre.

CONCLUSIÓN FINAL

La sobre-activación de señales de supervivencia en células humanas hepáticas, ya sea mediada por el TGF-β o por alteraciones moleculares concomitantes con el proceso tumorogénico, cambia el papel del TGF-β de supresor tumoral a promotor de tumores, impidiendo la muerte celular y promoviendo transiciones epitelio-mesenquima y la adquisición de un fenotipo de célula madre.

III. INTRODUCTION

1. Liver

1.1. Human liver anatomy and physiology

The liver is the largest internal organ in mammals, and carries out numerous functions that are involved in maintaining homeostasis within an organism. These functions include: 1) production of serum proteins, including clotting factors and transport proteins such as albumin and transferrin; 2) removal and breakdown of serum proteins, red blood cells and microbes; 3) production or removal of glucose during periods of fasting or eating, respectively; 4) processing of fatty acids and triglycerides; 5) maintaining cholesterol homeostasis via synthesis or catabolism; 6) synthesis and interconversion of non-essential amino acids; 7) breakdown of toxic endogenous compounds such as ammonia; 8) production and excretion of bile components; 9) detoxification of xenobiotic agents; and 10) the storage of numerous substances (Costa et al. 2003; Malarkey et al. 2005; Spear et al. 2006).

In broad terms, the liver consists of multiple units with an hepatic venule (also known as central vein) centrally surrounded by about 4-6 portal areas. Mice and rats each have 4 liver lobes: median (or middle), left, right, and caudate and all, except the left, are further subdivided into 2 or more parts. Mice and humans have a gall bladder, but not the rat. Human liver lobes have traditionally been designated as right, left, quadrate, and caudate, but it has been proposed that the liver can be subdivided into 9 segments based on the vascular and ductal branching patterns to the right and left sides (Malarkey et al. 2005). The adult liver is comprised of repeating structural units termed lobules. The lobule is defined as a hexagonal structure that consists of plates, which are hepatocytes, arranged in cords of one-two hepatocyte thick that extend from the periphery of the portal tracts to the central vein. The lobules are demarcated by peripheral spaces (portal spaces or portal triads) that contain small branches of the portal vein, the hepatic artery and the bile ducts. In the portal spaces, the terminal segments of the biliary system connect with hepatocytes in the liver parenchyma through the canals of Hering, narrow channels that are lined by hepatocytes and bile duct epithelial cells (BECs, also called cholangiocytes). The hepatocytes in the lobular plates are separated by sinusoids and the plates extend from the portal spaces to the central vein located at the center of the lobular structure (Fausto and Campbell 2003; Malarkey et al. 2005; Spear et al. 2006).

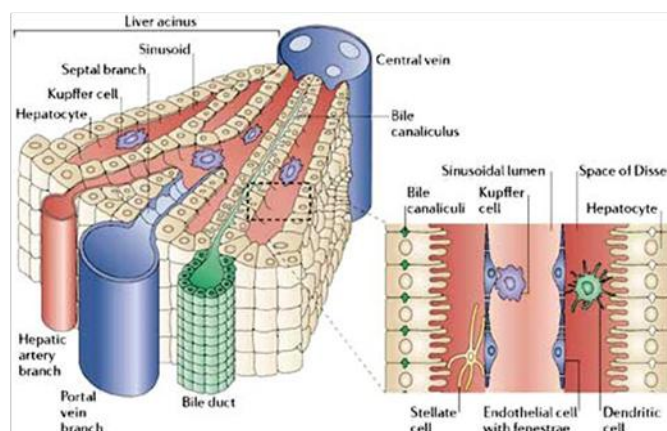


Figure 1. Hepatic lobule structure.

The portal vein and the hepatic artery are the two main vascular systems that supply blood to the liver. Blood from the terminal portal venules travels along plates of hepatocytes through small capillaries termed sinusoids. The hepatic artery generally accompanies the portal veins in the portal triads and its smaller branches feed the sinusoids at varying levels and the biliary tracts. The sinusoidal blood exits through central veins that converge into hepatic veins, which eventually lead to the vena cava. The sinusoids are lined by epithelial cells that form the barrier between the blood and hepatocytes; the narrow region between these two cell types is termed "space of Disse". The bile canaliculi, small channels that are between adjacent hepatocytes, transport bile to the intrahepatic bile duct in a

direction that is opposite to the sinusoidal blood flow. These intrahepatic bile ducts converge into larger extrahepatic ducts, which ultimately join the common bile duct that transports bile either to the gall bladder or directly to the small intestine (Malarkey et al. 2005; Spear et al. 2006). There is a compartmentalization of function determined by the position of hepatocytes within the liver lobule, a phenomenon called positional (or zonal) heterogeneity or metabolic zonation (Guyton and Hall 1999).

Cells from the canals of Hering function as stem cells or stem-like cells in the adult liver. These stem cells can generate small round cells referred to as oval cells in rodents (also called hepatoblasts, HB, in humans) which express markers of both fetal hepatocytes and biliary cells and are capable of generating hepatocytes and bile duct cells. Because of these properties, oval cells are considered to be bipotential progenitor cells in adult livers. Oval cells are thought to play an important role in liver regeneration, a remarkable property of the liver to regain its normal mass in response to parenchymal cell loss (Fausto and Campbell 2003; Spear et al. 2006).

The adult liver is comprised of numerous cell types a part from hepatocytes. Of these the principal ones are the followings: BECs that form the bile canaliculi. The sinusoidal endothelial cells (SECs) are the primary barrier between blood and hepatocytes and they filter fluids, solutes, and particles between the blood and space of Disse and represent up to 20% of the liver cells. SECs are a unique type of endothelial cells in that they have fenestrae, lack a basal lamina, and can transfer molecules and particles by endocytosis. Bone marrow-derived Kupffer cells, which represent 15% of the liver cells, are hepatic macrophages; these cells eliminate aged red blood cells and microbes, can present antigens and therefore influence immune function, and can produce a variety of cytokines and chemokines, which provide “cross-talk” with other cells. Hepatic stellate cells (HSCs) are another peri-sinusoidal cell type, which comprise about 5% of liver cells; these cells are the major reservoir for vitamin A and lipids in the body. HSCs produce a variety of extracellular matrix (ECM) proteins and can also synthesize numerous cytokines and chemokines; HSCs have a major role in the hepatic response to injury, and activation of HSCs due to chronic liver damage leads to fibrosis. Pit cells are the resident natural killer cells in the liver and are important in immune function (Guyton and Hall 1999; Malarkey et al. 2005; Spear et al. 2006).

1.2. Molecular mechanisms of liver differentiation

1.2.1. Liver development

At around embryonic day (E) 7.0 in the mouse, definitive endoderm emerges from the primitive streak to displace the extraembryonic endoderm of the yolk sac. Shortly after this, the endoderm invaginates to form a portal at the anterior region of the developing embryo that will ultimately define the foregut of the mouse. Primitive hepatic cells derived from the cranial part of the liver primordium proliferate, delaminate from the foregut endoderm, and invade the septum transversum mesenchyme. By approximately E8.0, the ventral wall of the foregut endoderm is positioned adjacent to the developing heart, and signals from the heart induce the underlying endoderm to initiate its development toward a hepatic fate (Costa et al. 2003; Zhao and Duncan 2005). By E9.5, the basement membrane surrounding the liver bud is lost, and cells delaminate from the bud and invade the surrounding septum transversum mesenchyme as cords of hepatoblasts. Hepatoblasts generate the two hepatic epithelial cell lineages, hepatocytes and cholangiocytes. The biliary epithelium derives from hepatoblasts located near vascular channels that will form the portal spaces of the mature liver lobules (Fausto and Campbell 2003; Zhao and Duncan 2005). The caudal part of the liver primordium then

gives rise to the extrahepatic bile ducts, the cystic duct and the gallbladder, which remains in continuity with the foregut and connect the liver hilum with the digestive tract. Moreover, the septum transversum mesenchyme contributes with endothelial cells and stellate cells that form and line the sinusoids. The hepatic vasculature develops as the liver bud grows and will ultimately help to establish the cellular architecture that is important for normal liver function (Spear et al. 2006). At around E10–11, hematopoietic stem cells originating from the aorta–gonads–mesonephros region (AGM region) colonize the fetal liver and expand their mass and lineage diversity. Along with the maturation of bone marrow and spleen around birth, hematopoiesis in the liver declines and hematopoietic stem cells migrate from the liver to these organs responsible for adult-type hematopoiesis. Meanwhile, many cell cycle regulated genes are silenced during this period and hepatic cells up-regulate the expression of numerous genes relating to the functions of mature liver in order to achieve their own metabolism after birth. The final step of hepatic differentiation takes place several days after birth in rodents (terminal differentiation). This process includes induction of another set of genes relating to functions of the adult liver, such as tryptophan oxygenase (TO) and several P450 species. In addition, cellular growth and expression of growth-related genes are either terminated or down-regulated during terminal differentiation. The lobular architecture is established during this period, along with zonal control of gene expression (Kinoshita and Miyajima 2002; Spear et al. 2006).

Liver development can be separated into several overlapping stages. In the first stage, when specification is established, cells become ‘competent’ and are capable of taking a certain fate but do not show any overt change. Competent cells subsequently become ‘committed’ to a particular lineage and exhibit morphological changes and express genes associated with commitment. Cells then ‘differentiate’ along that lineage and are ultimately capable of carrying out the functions of a terminally differentiated cell (Spear et al. 2006).

1.2.2. Liver development is controlled by different cytokines.

Liver gene induction in mouse endoderm starts when Fibroblast Growth Factor (FGF) production by the cardiogenic mesoderm is initiated. At this point, the developing heart is adjacent to the prehepatic endoderm and produces low amounts of FGF. Slightly later, the heart produces more FGF but becomes separated from the prehepatic endoderm by the septum transversum. To ensure that hepatic cells are not exposed to excessive concentrations of FGF, some morphogenetic events that move the endoderm away from the source of FGF take place (Serls et al. 2005). FGF signaling from the cardiogenic mesoderm is not sufficient for hepatic specification. In mouse embryos, the septum transversum produces bone morphogenetic protein (BMP)-2 and BMP-4, which cooperate with FGF to induce hepatic gene expression. The activity of BMPs is mediated by GATA-4, which contributes to the competence and specification of the prehepatic endoderm (Rossi et al. 2001; Huang et al. 2008). Wnt/β-catenin signaling is also involved in liver development: on one hand, it stimulates hepatoblast proliferation and lineage specification (Monga et al. 2003); on the other hand, it also controls growth of the liver and the acquisition of global liver morphology (Suksaweang et al. 2004). Other cytokines involved in liver development are Oncostatin M (OSM), Glucocorticoid, Hepatocyte Growth Factor (HGF) and Transforming Growth Factor-beta (TGF-β). OSM which is secreted from hematopoietic cells within the fetal liver not only contributes to control late stages of hepatocyte differentiation, possibly by increasing Hepatocyte Nuclear Factor 4α (HNF4α) expression, but also induces morphological changes, up-regulation of multiple liver-specific functions, ammonia, clearance, lipid synthesis, glycogen synthesis, detoxification and enhancement of homophilic cell adhesion.

Stimulation with glucocorticoid alone is capable of inducing most of the cellular responses of differentiation, however, to a far lesser extent than the combination with OSM (Kamiya et al. 1999; Kinoshita and Miyajima 2002). Hepatocyte growth factor (HGF) is expressed by the septum transversum, the endothelial cells, and the hepatoblasts; and its receptor, c-met, is found at the surface of hepatoblasts, where it promotes proliferation (Lemaigre 2009). The TGF-β/Smad2/Smad3 pathway also stimulates proliferation, because mice with heterozygous mutations in the *Smad2* and *Smad3* genes showed liver hypoplasia; in vitro, these effects can be reversed by HGF (Weinstein et al. 2001). It is likely that the HGF and TGF-β pathways converge on the β1-integrin expression, which is necessary for proliferation (Fassler and Meyer 1995).

1.2.3. Liver development and differentiation are tightly regulated by different transcription factors.

Different reviews have focused their attention on the role of the different transcription factors (TF) involved during liver differentiation; here we highlight the most relevant ones. The Foxa (Forkhead box a) family is comprised of three members, Foxa1, Foxa2 and Foxa3 (formerly HNF3α, HNF3β and HNF3γ), they are known to bind the albumin enhancer prior to the onset of albumin expression; in addition, they also bind to transthyretin and to α-1-antitrypsin promoters (Costa et al. 2003; Spear et al. 2006). The ONECUT family is comprised of HNF6 or ONECUT-1 (OC-1) and a second member called OC-2, which is also expressed in the liver and shares DNA-binding site specificity and homology with the HNF6 protein and may provide functional redundancy with HNF6 in hepatocytes. HNF6 is essential for regulating expression of HNF1β that plays an important role in development of the gallbladder and IHBD (intrahepatic bile ducts) (Costa et al. 2003). Interestingly, TGF-β promotes differentiation of hepatoblasts to biliary cells and represses hepatocyte differentiation: the transcription factors HNF6 and OC-2 are expressed in hepatoblasts, hepatocytes and cholangiocytes, but the highest levels are found in the latter. Moreover, HNF6 and OC-2 control hepatoblast differentiation, blocking TGF-β signaling activity allowing hepatocyte differentiation (Clotman et al. 2005), therefore HNF6 controls the timing of hepatoblast fate decision. SOX9 is downstream of HNF6 and its function is to repress C/EBPα expression, which seems to be needed for normal duct development (Antoniou et al. 2009). Another important TF in the liver is HNF1α, which is co-expressed with the isoform HNF1β and they can form heterodimers (Costa et al. 2003). HNF1α plays an important role in the transcriptional activation of differentiated hepatocyte-specific genes critical for liver function but is not required for specification of the hepatocytic cell lineage (Pontoglio et al. 1996). In mouse development, HNF4α is expressed in the primary and extra-embryonic visceral endoderm prior to gastrulation and in epithelial cells at the onset of liver, pancreas, and intestine formation (Duncan et al. 1994). HNF4α is critical for regulating transcription of genes involved in gluconeogenesis and glycogen synthesis, postnatal lipid, cholesterol, bile acid homeostasis and in cell adhesion required for epithelial cell morphology (Li et al. 2000; Parviz et al. 2003).

The CCAAT/enhancer binding proteins (C/EBP), C/EBPα and C/EBPβ, are co-expressed in hepatocytes and are able to form either homodimers or heterodimers. C/EBPα regulates expression of genes involved in hepatic glucose and lipid homeostasis as well as negatively regulating hepatocytes proliferation (Costa et al. 2003). The GATA factors comprise a family of transcriptional regulators, of which GATA-4 and GATA-6 are of particular interest in regards to liver gene regulation, as they are involved in the regulation of albumin and HNF4α expression, respectively during hepatoblast differentiation (Spear et al. 2006). The hepatocyte nuclear factors HNF1α and β, c/EBPα, HNF4α,

and Foxa, act in combination to control aspects of hepatocyte differentiation and liver function. Of these transcriptional regulators, the nuclear hormone receptor HNF4α appears to be very potent in controlling hepatocyte differentiation. HNF4α plays a fundamental role in transforming the fetal liver into an epithelial parenchyma during embryogenesis. In addition to controlling hepatocyte differentiation during embryogenesis, HNF4α is also required to maintain a differentiated hepatocyte phenotype (Lemaigre and Zaret 2004; Zhao and Duncan 2005).

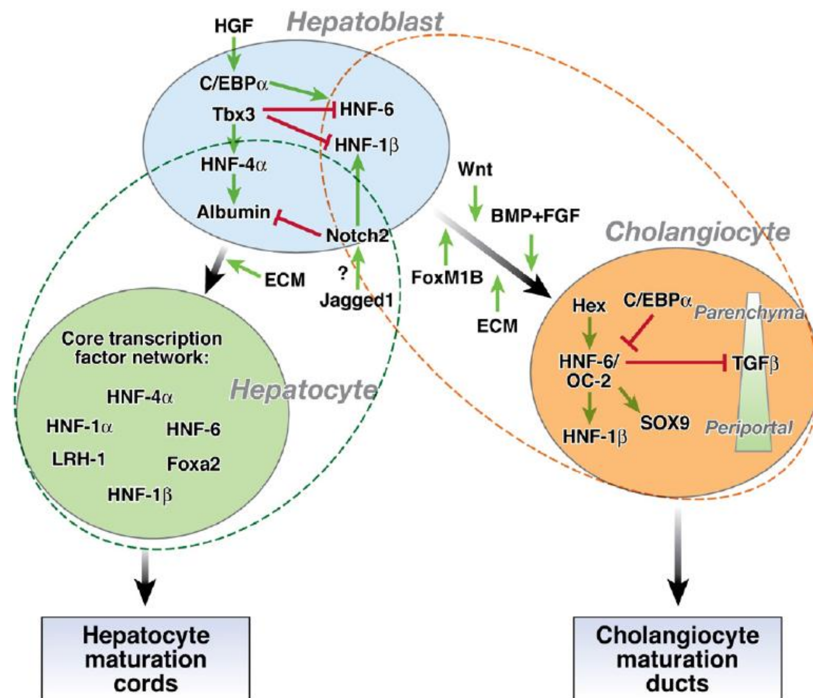


Figure 2. Mechanisms of cell differentiation, adapted from Lemaigre et al 2009.

In the adult liver, these transcription factors have key roles in liver functions. HNF1α and HNF4α control glucose metabolism as well as several other hepatic functions such as lipid and amino acid metabolism. HNF1β is essential for bile acid sensing and fatty acid oxidation. The three Foxa factors have overlapping DNA-binding properties and, as the other liver-enriched factors, regulate numerous hepatic functions. HNF6 mediates some effects of growth hormone, inhibits glucocorticoid activity, and stimulates expression of genes in the gluconeogenic, glycolytic, and bile acid synthesis pathways as well as hepatocytes proliferation. C/EBPα regulates glucose and glycogen metabolism as well as lipid homeostasis and hepatocytes proliferation. Another member of the family, C/EBPβ, is a regulator of gluconeogenesis and a potent stimulator of phosphoenolpyruvate carboxykinase (Spear et al. 2006; Lemaigre 2009).

1.3. Liver stem cells

Stem cells are generally defined as cells exhibiting two properties: a capacity for self-renewal and potency for multilineage differentiation. They are the source of progenitor cells committed to one or several lineages. The committed progenitor cells exhibit a capacity for active proliferation and supply abundant daughter cells, which in turn give rise to terminally differentiated cells. Stem cells are classified into three categories: totipotent, pluripotent, or multilineage somatic. Totipotent stem cells are capable of generating a fetus in utero by themselves over the course of ontogeny. Stem cells lacking potency for ontogeny are excluded from this category. Pluripotent cells are embryonic stem (ES) cells which are capable of differentiating into cells derived from any of the germ layers, including germ line cells; however, they cannot generate a fetus without an interaction between themselves and a fertilized egg. Other sources of pluripotent cells are induced pluripotent stem cells (iPS cells, which exhibit characteristics similar to those of ES cells) and mesenchymal stem cells (MSCs). Somatic stem cells, by contrast, do not generally exhibit a capacity to differentiate into any

cell type other than their own tissue (Roskams 2006; Kakinuma et al. 2009). Somatic stem cells are expected to display certain characteristics: (1) self-renewal, (2) multipotentiality, (3) transplantability and (4) functional long-term tissue reconstitution. Stem cells themselves are required to maintain their undifferentiated state while dividing. In contrast, progenitor cells show a limited ability to self-renew, and they comprise distinct subpopulations with variable lineage potential. Moreover, unlike stem cells, progenitor cells divide rapidly but cannot be serially transplanted and hence have been named transit amplifying cells (Shafritz et al. 2006). Definitions for the liver stem cell include the following: (1) cells responsible for normal tissue turnover, (2) cells that give rise to regeneration after partial hepatectomy, (3) cells responsible for progenitor-dependent regeneration, (4) cells that produce hepatocyte and bile duct epithelial phenotypes in vitro, and (5) transplantable liver-repopulating cells (Duncan et al. 2009).

1.3.1. Stem Cell Location

The human liver contains two pluripotent progenitors: hepatic stem cells (hHpSCs) and hepatoblasts (Zhang et al. 2008). Hepatic stem cells in fetal and neonatal livers have been found recently to be located in the ductal plates. These cells give rise to the rapidly proliferative, transit amplifying cells-hepatoblasts, the dominant cell type of fetal and neonatal livers. Hepatoblasts, in turn, give rise to the hepatocytic and biliary lineages, the hepatocytes and cholangiocytes. The ratio of hepatoblasts to differentiated cells changes depending on the developmental stage, with the hepatoblasts being dominant in fetal and neonatal livers and with few if any mature parenchymal cells. By contrast, in pediatric and adult human livers, the hepatic stem cells are the dominant pluripotent progenitor (0.3%–0.7%); the hepatoblasts are few (<0.1%), and the majority (>98%) of the parenchymal cells are diploid and polyploid hepatocytes and biliary epithelia (Schmelzer et al. 2006). Interestingly, hHpSC persist in stable numbers throughout life, constituing 0.5% to 2.5% of liver parenchyma of the liver at any given age (Schmelzer et al. 2006; Schmelzer et al. 2007). In pediatric and adult livers, hHpSCs and hepatoblasts are found as individual cells or small clusters of cells tethered to the ends of the Canals of Hering (Roskams et al. 2003; Zhang et al. 2008). Other groups have identified four possible hepatic stem cell niches in the liver: the Canal of Hering (proximal biliary tree), intralobular bile ducts, periductal “null” mononuclear cells, and peribiliary hepatocytes (Kuwahara et al. 2008).

During and after episodes of severe liver injury in both human and animal liver, a considerable proportion of the cells in the hepatic progenitor cell compartment and of mature hepatocytes and cholangiocytes are derived from hematopoietic stem cells. Thus, it seems that the bone marrow forms a reservoir which is appealed to when there is extensive hepatic stem cell activation (Libbrecht and Roskams 2002). Hepatocytes derived from bone marrow cells could be formed by three primary mechanisms. First, bone marrow could theoretically harbor specialized endodermal stem cells that are capable of producing hepatocytes and other epithelial cells (Petersen et al. 1999). Secondly, a single stem cell population could give rise to the blood and hepatocyte lineages (Theise et al. 2000). Thirdly, in contrast to differentiation, bone marrow–derived hepatocytes could be derived by cell fusion (Quintana-Bustamante et al. 2006). However, it is widely believed that hepatocytes derived from the bone marrow play a minor role, if any, in therapeutic liver repopulation (Duncan et al. 2009). Finally, liver stem cell population can be replenished by bone marrow-derived stem cells. Wulf et al showed that transplanted bone marrow hematopoietic stem cells, purified on the basis of Hoechst dye efflux, generate hepatic stem cells (SP cells) and ultimately form hepatocytes and biliary epithelium (Wulf et al. 2003).

Multipotent stem/progenitor-like cells have been isolated from tissues outside the liver, and in some cases hepatocytic differentiation has been reported. These cells are unlikely to serve as liver stem cells within the organism but nonetheless are of therapeutic and biomedical interest. Recently there have been different reviews that have focused on the use of stem cells from extra-hepatic origin for hepatic regeneration (Alison et al. 2009; Duncan et al. 2009). **Pluripotent adult stem cells** isolated from multiple tissues, including bone marrow, umbilical cord and umbilical cord blood (UCB), amniotic fluid and fetal liver among others; and **mesenchymal stem cells** (MSCs) isolated from bone marrow or fat can be differentiated into hepatocyte-like cells in vitro using differentiation protocols that include the use of multiple factors such as dexamethasone, Epidermal Growth Factor (EGF), HGF, FGF1/4, and OSM. **Embryonic stem cells** (ESC) can be differentiated towards the hepatic lineage. Most published ESC differentiation protocols generate hepatocyte-like cells, but not the fully functional, mature hepatocyte. ESCs are allogeneic in nature, and transplantation of hepatocyte-like cells generated from ESC will require immunosuppression; furthermore, ethical concerns may constitute limitations for their use. The newly described induced pluripotent stem cells or iPS cells, generated by introduction of OCT4, SOX2, c-MYC and KLF4 or OCT3/4, SOX2, NANOG and LIN28 in terminally differentiated cells, display many if not all features of ESC and might circumvent the problems due to HLA-mismatching invariably associated with ESC based therapies. Finally, some studies have indicated that **hepatocytes** are bipotential. Fully differentiated hepatocytes are efficient in liver repopulation and have stem cell–like capacity for cell division; different studies have shown that mature hepatocytes are serially transplantable liver-repopulating cells (Overturf et al. 1997; Weglarz et al. 2000). In the same line of evidence, in vitro experiments have shown hepatocyte differentiation into ductular epithelium (Limaye et al. 2008). Moreover, liver repopulation has been shown to be mediated by non-hepatocyte cell types such as fetal hepatoblasts (Mahieu-Caputo et al. 2004), oval cells (Suzuki et al. 2008), pancreatic liver progenitors (Wang et al. 2001), MSCs from the umbilical cord (Yan et al. 2009) or from adipose tissue (Banas et al. 2008), and Hematopoietic Stem Cells (Lagasse et al. 2000). However, increasing evidence suggests that the use of MSCs for hepatic regenerative medicine may promote liver cirrhosis (di Bonzo et al. 2008).

1.3.2. Liver Stem Cell Markers

There is an important debate within the liver community on establishing markers that would discriminate hepatic stem cells from hepatoblasts, hepatocytes and cholangiocytes. The human counterparts to the oval cells described in rodents are often referred to as hepatic progenitor cells (HPC) or hepatoblasts. It has also been possible to isolate multipotent progenitor cells or hepatic stem cells from fetal, neonatal and adult liver (Dan et al. 2006; Schmelzer et al. 2006; Inada et al. 2008). Oval cells or HPC are described as small epithelial cells with an oval nucleus and scant cytoplasm which are bipotential cells that can differentiate towards the biliary and the hepatocytic lineage. Differentiation towards hepatocytes occurs via intermediate hepatocyte-like cells, while differentiation towards the biliary lineage leads to the formation of atypical reactive ductules (Libbrecht and Roskams 2002). Oval cells express phenotypic markers of both (immature) hepatocytes (such as α-fetoprotein, albumin and CK8, 18) and bile duct cells (such as bile duct–type cytokeratins [CK7, 19]), they are as well strongly immunoreactive to rat oval cell marker OV-6, and several stem-cell-related markers associated with hematopoietic stem cells, including c-Kit, CD34, CD90 (Thy-1), LIF (Leukemia Inhibitory Factor), Sca-1, in addition to mRNA for flt-3 (Roskams et al. 2003; Kakinuma et al. 2009). Consequently, it has been suggested that oval/progenitor cells may have an hematopoietic origin (Terrace et al. 2007). In addition to their in vitro hepatocytic and biliary

differentiation capacities, these cells could express multiple pancreatic markers after long-term culture or transplantation to an in vivo pancreatic environment (Duncan et al. 2009). Other groups have described hepatic stem cells with a different expression profile. Dr. Fausto's group has described hepatic stem cells to have a high self-renewal capability with their immunophenotype being: CD34+, CD90+, c-kit+, EpCAM+, c-met+, SSEA-4+, CK18+, CK19+, albumin-, α-fetoprotein-, CD44h+, and vimentin+; these hepatic stem cells are mesenchymal-epithelial transitional cells, probably derived from the mesoendoderm and have the ability to differentiate into hepatocytes and bile duct cells, as well as into fat, bone, cartilage, and endothelial cells (Dan et al. 2006). Suzuki et al. showed that a single cell in the c-Met+, CD49f+/low, c-Kit−, CD45− and Ter119− fraction from mid gestational fetal liver has the capacity for self-renewal in vitro and for bipotential differentiation, indicating that this defined fraction contains hepatic stem cells (Suzuki et al. 2002).

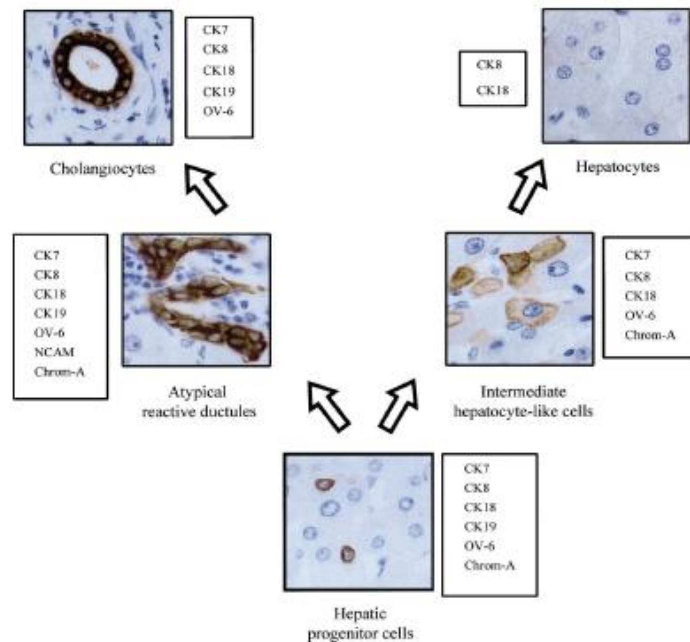


Figure 3. Differentiation of Oval cells. Adapted from Libbrecht and Roskams, 2002.

Other groups focus their attention towards the description of markers that permit the selection of hepatic stem cells. EpCAM is highly expressed in many human cancers with an epithelial origin. Epithelial cell adhesion molecule (EpCAM), a 34–40-kDa transmembrane glycoprotein is expressed by hepatic stem cells, hepatoblasts, and committed progenitors but not by mature hepatocytes. EpCAM have lately become one of the main protein markers used to sort progenitor cells in livers (Inada et al. 2008; Zhang et al. 2008). The Reid group has described hepatic stem cells and hepatoblasts expression profiles thanks to their isolation by sorting EpCAM + cells. Hepatic stem cells (HpSCs) isolated from the livers of all developmental stages expressed very similar gene

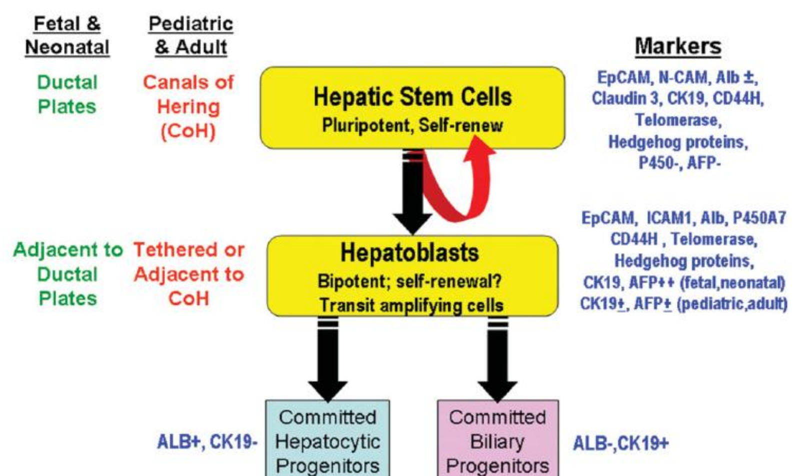


Figure 4. Markers for hHpSC and hHB and the location of cells with those markers within the liver acinus. Adapted from Zhang et al 2008.

expression profiles, with high expression of CK19, EpCAM, neuronal cell adhesion molecule (NCAM), CLDN-3, c-kit, CD44H, CD133/1, E-cadherin, N-cadherin, CK8 and 18, integrin-α1 and aquaporin 4 but low expression levels of albumin and very low or no expression of AFP (alfa-fetoprotein), intercellular adhesion molecule (ICAM)1 or of any liver-specific genes (e.g., connexins, PEPCK, DPP4, cytochrome P450 3A4, and transferrin). A minor HpSCs cells subpopulation expresses CD117 (c-kit). Additionally, HpSCs have self-renewal capacity. Finally, HpSCs are negative for hemopoietic markers, endothelial cell markers, and mesenchymal markers, such as those for hepatic stellate cells. Gene expression patterns of hepatoblasts are different from those of HpSCs. They have lost the expression of CLDN-3 and NCAM, present low expression of genes characteristic of the stem cell phenotype, such as CK19 and c-kit, low levels of adult liver-specific genes (e.g., connexins, PEPCK, and P450s) and intense expression of AFP, a defining feature of hepatoblasts. Hepatoblasts also express ICAM1, CD133/1, and CD44H. However, hepatoblast do not show self-renewal capacity. Hepatocytes from postnatal livers showed the absence of expression of the genes defining the hepatic stem cells (e.g., NCAM, CK19, c-kit, and CLDN-3) or hepatoblasts (e.g., AFP) and high levels of expression of classic liver-specific genes such as albumin, connexins, transferrin, PEPCK, DPP4, P450s (as cytochrome P4503A4) (Schmelzer et al. 2006; Schmelzer et al. 2007). EpCAM+ fetal liver cells possess the capacity for multilineage gene expression, including expression of Oct4, Nanog and alkaline phosphatase, which are markers of pluripotency in hESCs (Inada et al. 2008); these results are in accordance with the ones presented before from Fausto group in which they showed that hepatic stem cells have the ability to differentiate into a variety of epithelial and mesenchymal lineages (Dan et al. 2006). Additionally, Dr. Mishra's group has described two to four cells out of the entire 30,000 to 50,000 cell population of living donor liver-transplanted specimens that expressed (Signal Transducers and Activators of Transcription) STAT3, Oct4, and Nanog and TGF-β signaling proteins, TGF-beta Receptor II (TβRII) and ELF. These cells also stained positively for both hepatocytic and cholangiocytic cell lineage marker, albumin and cytokeratin-19, respectively. These putative progenitor/stem cells were generally found localized in the portal tract region surrounded by a ‘‘shell’’ of six to seven cells expressing TβRII, ELF, and albumin, but not NANOG or OCT4, reflecting a more differentiated phenotype (Tang et al. 2008).

A common defense mechanism adopted by stem cells is their high expression of ABC membrane transporters. This property was exploited to isolate Hematopoietic Stem Cells based on their ability to efflux Hoechst 33342 dye; after fluorescence activated cell sorting (FACS) analysis, those hematopoietic cells with the ability to actively efflux the Hoechst dye appear as a distinct population of cells known as the “side population” (Goodell et al. 1996). Hepatic Side Population (SP) cells expressed stem cell markers CD34, c-kit, Sca-1 and Thy-1, on both CD45+ and CD45- cells. Hepatic SP cells present characteristics of both hepatocyte and cholangiocyte lineages. Moreover, SP cells isolated from Hepatocellular Carcinoma (HCC) cell lines have higher expression of stemness genes (Wulf et al. 2003; Chiba et al. 2006).

Thy1 has also been used as a powerful marker of hepatic stem cells. Thy-1+ cells are mainly found in the portal tract and the surrounding parenchyma. Thy-1+ cell populations are positive for progenitor (CD34, c-kit, CK14, M2PK-the fetal M2 isoform of pyruvate kinase, OV6, nestin, chromogranin A), biliary (CK19) and hepatic (Hepar1) markers revealing their progenitor as well as hepatic and biliary nature, they are also positive for CD45, an hematopoietic marker. Thy+ cells can be differentiated towards the hepatocytic lineage and successfully engrafted in immunodeficient mice livers (Weiss et al. 2008).

1.3.3. Activation of the stem cell compartment

Hepatocytes in normal adult liver hardly ever proliferate and have a life span of over a year. After partial hepatectomy however, proliferation of the main epithelial compartments (hepatocytes and cholangiocytes), followed by proliferation of the mesenchymal cells (hepatic stellate cells and endothelial cells), quickly restores the liver. When the mature epithelial cell compartments of the liver, hepatocytes and/or cholangiocytes are damaged or inhibited in their replication, a reserve cell compartment is activated (Roskams 2006; Weiss et al. 2008). Proliferation and differentiation of hepatic progenitor cells is referred to as 'activation' and this process occurs to a variable degree in almost all human liver diseases such as acute liver necrosis, hemochromatosis, chronic cholestatic diseases, alcoholic liver disease and chronic viral hepatitis (Libbrecht and Roskams 2002). The activation of the stem cell compartment is referred to as a "ductular reaction" in humans and "oval cell reaction" in rodents. This process involves expansion of bipotential transit amplifying progenitor cells, which can differentiate into hepatocytes and biliary cells (Mishra et al. 2009). The degree of stem cell and intermediate hepatocyte activation correlates with the degree of inflammation and fibrosis in diseases such as chronic hepatitis, hemochromatosis, and nonalcoholic steatohepatitis (Roskams et al. 2003). As recently reviewed, the oval cell response can be divided into 4 phases: activation, proliferation, migration, and differentiation; several factors are involved in this response, including TGF-β, HGF and its receptor c-met, the plasminogen activator/plasmin system, interleukin 6 (IL6) and peroxisome proliferator activated receptor alpha (PPARα), Interferon-γ (IFN-γ) and TGF-β (Duncan et al. 2009).

The existence of a hepatic stem cell compartment gives rise to expectations regarding their practical applications. Understanding and identification of the hepatic stem cells and hepatoblasts may provide for new therapeutic treatments to liver pathologies such as congenital metabolic diseases, fibrosis, end-stage liver cirrhosis, and hepatocarcinogenesis. Human hepatic stem cells most likely can give rise to HCC as well as cholangiocarcinomas.

2. HCC: incidence, current treatments and deregulated pathways

2.1. HCC incidence, origin and treatment

As recently reviewed, HCC is the fifth most common cancer in the world and the third cause of cancer-related death globally, in addition HCC accounts for 70-90% of human liver cancers. Most HCCs (80%) arise in a cirrhotic liver, a situation where there has been long-standing hepatocyte damage and chronic inflammation leading to fibrosis. There are huge geographical variations in the incidence of HCC, with the highest incidence in areas such as eastern Asia and sub-Saharan Africa where chronic hepatitis B virus (HBV) infection is a major risk factor. In Europe and USA, the incidence of HCC is low but slowly increasing, probably as a result of the rise in people infected with hepatitis C virus (HCV) (Llovet and Bruix 2008). Apart from hepatotrophic viruses, the other major risk factors for HCC are conditions leading to cirrhosis such as alcohol abuse and metabolic liver disease, and mutagens such as aflatoxins, toxic metabolites of the food mould *Aspergillus* sp. (Alison and Lovell 2005). Another type of liver cancer are cholangiocarcinomas (CC), which are believed to arise from biliary epithelium cells (Alison et al. 2009).

It has been thought for a long time that the mature hepatocyte represents the cell of origin of all hepatocellular carcinomas (HCCs). However, about half of the human hepatocellular carcinomas express one or more markers of progenitor cells such as α-fetoprotein, CK7, CK19, and CK14 among others. This expression profile can be the result of the acquisition of progenitor cell markers during malignant transformation of either mature hepatocytes (dedifferentiation hypothesis) or progenitor cells that are differentiating toward the hepatocytic lineage (maturation-arrest hypothesis) (Libbrecht and Roskams 2002). Half of the small cell dysplastic foci, the earliest precursor lesions, consist of progenitor cells and intermediate hepatocytes, suggesting that these lesions are the result of activation and proliferation of progenitor cells (Roskams et al., 2003). Accordingly, HCC is also likely to possess the so-called cancer stem cells (CSCs), otherwise known as tumor-initiating cells, although their identity is far from clear, with the side population (SP), CD133, OV6 and Thy-1 (CD90) being proposed as markers for these cells (Alison et al. 2009). Interestingly, in different HCC cell lines there is a small percentage of cells that are OV6 positive. These OV6+ subpopulation, not only expresses higher levels of progenitor cell markers, such as ABCG2, EpCAM, c-kit, AFP, as well as transcription factors in the earlier phase of hepatic development, including GATA6, C/EBPα/β; but also has an increased expression of ‘‘stemness’’ genes, including Notch-1, Bmi1, Nanog, and Oct-4. Finally, β-catenin signaling was increased in OV6+cells, being required for protection of OV6+ progenitor-like cells from chemotherapeutics-induced cytotoxicity (Yang et al. 2008c). In another study, it was observed that freshly isolated CD133+ cells from HCC cell lines possess similar characteristics to those of stem/progenitor cells, including greater colony-forming efficiency, higher proliferative output, greater ability to form tumor in vivo, ability for self-renewal, and ability to differentiate into non-hepatocyte-like, angiomyogenic-like lineages, and expressed stemness genes (Ma et al. 2007b). However, at the same time, it is also believed that HCC, like many other cancers, develops from the accumulation of mutations in genes critical to processes such as self-renewal, cell growth, and other functions. It has been estimated that approximately 3 to 6 genetic events are necessary to transform a normal cell into a cancer cell (Hahn and Weinberg 2002). Altogether, these results suggest that both dedifferentiation of mature hepatocytes and maturation arrest of progenitor cells may lead to hepatocellular carcinoma in humans.

Regarding the differentiation state of liver cancer cells different groups have proposed possible classifications of HCC. EpCAM is highly expressed in many human cancers with an epithelial origin. EpCAM is an early biomarker of HCC because its expression is highly elevated in premalignant hepatic tissues and in a subset of HCC: ~ 35% of HCC cases express EpCAM. Likewise, AFP, a known HCC prognostic factor, is expressed in embryonic liver and is silent in adult liver, but ~60% of HCC patients have elevated AFP in the serum. EpCAM+ HCCs had an elevated expression of HPC markers (c-Kit, CK19, CD133) and an activation of β-catenin. Interestingly, HCC can be classified in four subtypes based on EpCAM expression and on patients' serum level of AFP. Each of these subtypes had a unique expression pattern with features resembling various stages of hepatic lineages: those with the poorest prognosis possessed a higher proportion of either EpCAM+/AFP+ cells (hepatic stem cell-like) or EpCAM−/AFP+ cells (hepatic progenitor cell/oval cell-like); whereas those with EpCAM−/AFP− cells (mature hepatocyte-like) or EpCAM+/AFP− cells (cholangiocyte-like) had a more favorable outcome (Yamashita et al. 2008b). Another classification based on HCC cell origin was proposed by the Thorgeirsson group, by applying hierarchical clustering analysis of gene expression patterns, they identified a new prognostic subtype of HCC named HB that shares gene expression patterns with fetal hepatoblasts, as they have a significantly higher level of expression of CK7 and CK19. They argue that the unique expression profile of HB subtype could be the result of malignant transformation of mature hepatocytes with concomitant dedifferentiation that results in the acquisition of progenitor-cell features or from HPC origin (Lee et al. 2006a).

Regarding the prognostic of HCC, there are several prognostic scoring systems including the Barcelona-Clinic Liver Cancer (BCLC), the Cancer of the Liver Italian Program, the Chinese University Prognostic Index and the Japanese Integrated Staging. They use different permutations of variables related to the severity of liver disease, number and size of tumor nodules, and cancer spread. Although there is not one universally accepted HCC staging system, many have adopted the BCLC group's proposal of 5 stages, further validated in a large North American experience. The BCLC's staging and prognostic system accounts for variables related to tumor stage, physical and liver functional status, and cancer-related symptoms and also provides a link to a treatment algorithm. The Child–Turcotte–Pugh (CTP) class, which provides an assessment of the synthetic function, may serve complementary to the BCLC staging in providing a more refined treatment algorithm. The Barcelona Clinic Liver Cancer (BCLC) clinical classification has been endorsed by the European Association for the Study of the Liver as well as the American Association for the Study of the Liver Diseases as the standard guideline for the clinical management of HCC patients (Llovet et al. 2004).

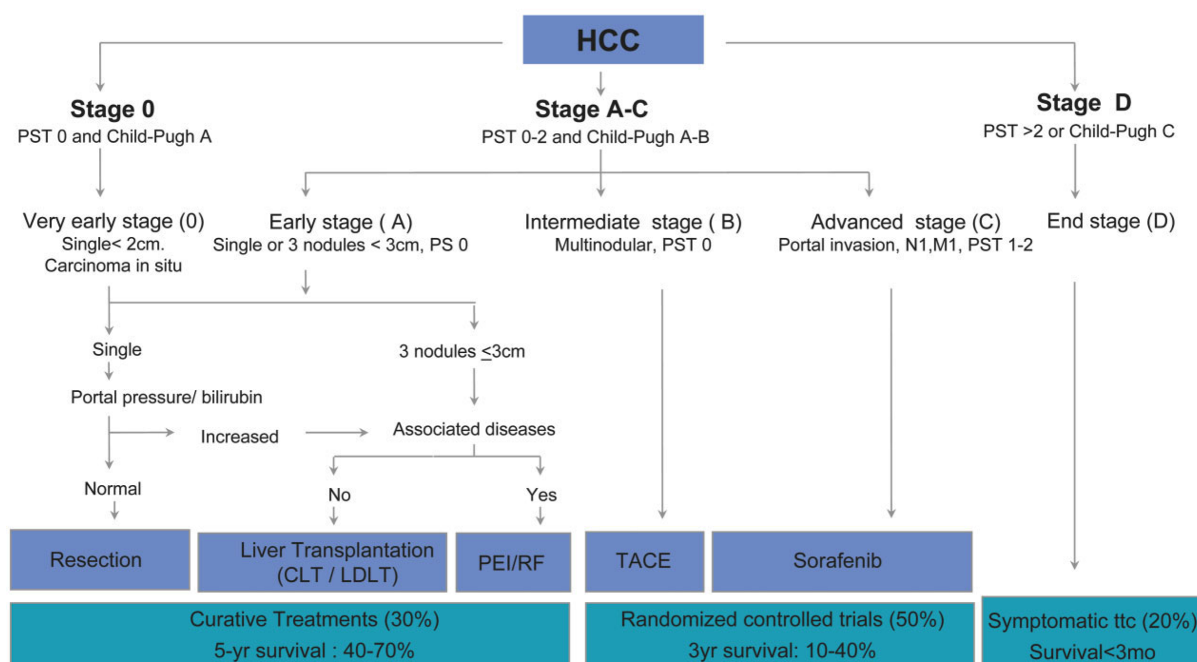


Figure 5. BCLC staging classification and treatment schedule, from Llovet and Bruix, 2008.

Currently, medical therapy is not effective in treating most HCC, and the only hope of cure is either resection or liver transplantation, with a 5 year survival rate of less than 5% that correlates with tumor staging at the time of diagnosis. Less than 40% of patients are eligible for curative treatments, as the majority of HCC patients present with an advanced stage of cancer and are often inoperable because of extensive tumor involvement of the liver or underlying hepatocellular disease. The use of chemotherapy and radiotherapy have limited efficacy (Vander Borght et al. 2008; Yang et al. 2008d). In the last 25 years, many antitumoral agents have been evaluated in patients with unresectable HCCs; there are many randomized controlled clinical trials (RCT) assessing transarterial chemoembolization (TACE), intra-arterial and systemic chemotherapy, hormonal treatments, immunomodulators, and internal and external radiotherapy. Only chemoembolization with gelfoam and doxorubicin or cisplatin when compared with control or subobtimal therapies has shown increased survival. Nowadays, TACE is considered the standard treatment for patients with intermediate stage cancer. In 2006, no drug was effective as first-line treatment for patients with advanced HCC which represent 40%-70% of the whole HCC population. Fortunately, the absence of standard systemic therapy for advanced cases has changed with the recent positive RCT testing the multikinase inhibitor Sorafenib, which represents a breakthrough in the management of this neoplasm (Llovet et al. 2008). Sorafenib (BAY 43-9006; Nexavar, Bayer Pharmaceuticals, West Haven, Conn) is an oral multikinase inhibitor that blocks tumor cell proliferation by targeting the Raf/mitogen-activated protein kinase/extracellular signal regulated kinase (Raf/MEK/ERK) signaling pathway and exerts an antiangiogenic effect by targeting the tyrosine kinases (TKs), VEGFR-2, VEGFR-3, and PDGFR-β (Wilhelm et al. 2006). Sorafenib exhibited growth-inhibitory effects, induction of apoptosis, and down-regulation of the anti-apoptotic protein MCL1 through a Raf/MEK/ERK-independent mechanism (Liu et al. 2006).

2.2. Molecular mechanisms of HCC

According to Volgestein, solid tumors develop after disruption of at least three critical intracellular signaling networks, whereas hematological tumors develop after only one genomic hit, as in chronic myeloid leukemia. Disruption of specific pathways results from oncogene activation (generally one allele activation suffices) through point mutations, copy number alterations, or epigenetic changes; or through inactivation of tumor suppressor genes (generally inactivation of two alleles required) resulting from loss of heterozygosity, point mutations or epigenetic silencings (Vogelstein and Kinzler 2004). Hanahan and Weinberg proposed that signaling pathway disruption in cancer can be grouped into six function capabilities, some of which need to be altered for cancer development: self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tumor invasion and metastases (Hanahan and Weinberg 2000). In addition to these six hallmarks, additional hallmarks of cancer cellular phenotypes have been proposed, these cancer phenotypes are not responsible for initiating tumorigenesis, but they are common characteristics of many tumor types; they consist of DNA damage/replication stress, proteotoxic stress, mitotic stress, metabolic stress, and oxidative stress that would constitute the stress phenotypes of cancers (Luo et al. 2009).

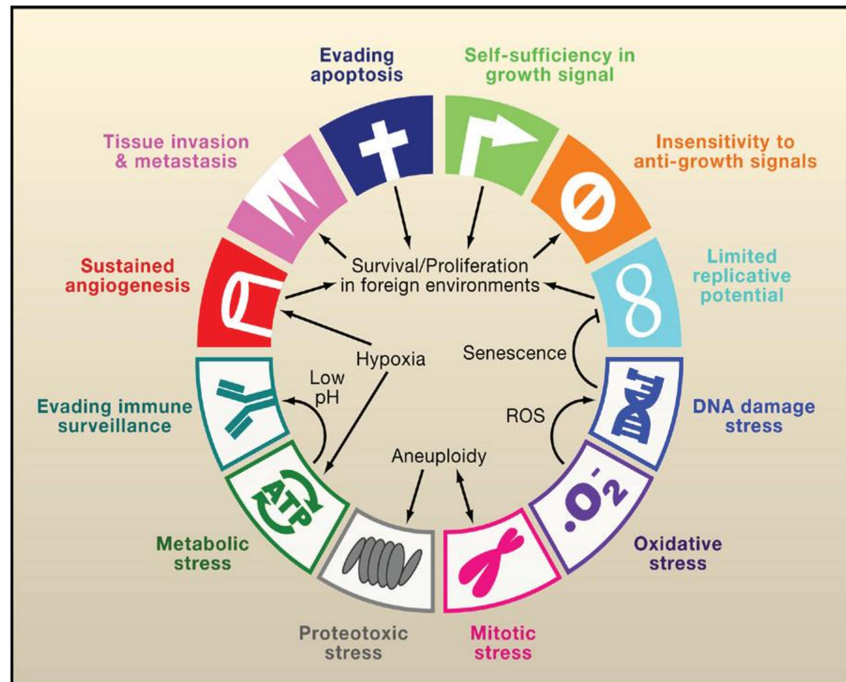


Figure 6. Proposed Hallmarks of Cancer by Luo et al 2009.

The development of HCC is a multi-step process often beginning with fibrosis/cirrhosis, progressing to adenoma and dysplastic nodule formation (Feitelson et al. 2002). HCC is typically a hypervascular tumor, dependent on neo-angiogenesis, the formation of new blood vessels from preexisting vascular beds, to receive an adequate supply of oxygen and nutrients (Sun and Tang 2004). Moreover, angiogenesis is necessary for tumor invasiveness and metastasis (Baek et al. 2008). The accumulation of genetic alterations driving a cirrhotic liver to cancer is a multistep process originating from stem cells or mature hepatocytes. Although the underlying molecular mechanisms of HCC pathogenesis remain largely unknown, multiple epigenetic and genetic changes have been associated with HCC, including the activation of oncogenes (e.g., N-RAS, c-myc, c-fos) and inactivation of tumor suppressor genes (e.g., p53, p16, Rb) (Thorgeirsson and Grisham 2002; Aravalli et al. 2008). For instance, as it has been recently reviewed, HCV and HBV are critical insults for genetic damage. In patients with chronic HCV infection, an increase in TGF-α and insulin-like growth factor-2 (IGF-2) contribute to accelerate hepatocyte proliferation. The HCV core protein acts as a Wnt ligand, transactivates RAS signaling, and inactivates p53. In chronic HBV infection, non-random DNA

integration of HBV leads to promoter activation of oncogenes, DNA rearrangement, and chromosomal instability (Llovet and Bruix 2008). A significant number of relevant molecular mechanisms altered in HCC initiation and progression compromise the balance between survival and apoptotic signals in the pre-neoplastic hepatocytes or hepatic progenitor cells. Some physiological pro-apoptotic molecules are down-regulated or inactivated in HCC, but the balance between death and survival is mainly disrupted due to overactivation of anti-apoptotic signals (Fabregat et al. 2007). In this section, I will address the most common deregulated pathways which have been recently described for HCC.

Cell Cycle regulation. The CDK inhibitors p16INK4A, p21(WAF1/CIP1), and p27Kip1 are independently affected and a change in the expression of one or more of these inhibitors contributes to carcinogenesis in nearly 90% of HCC cases (Azechi et al. 2001). Furthermore, several studies have demonstrated that the pRb pathway is severely disrupted in HCC patients (Farshid et al. 1994; Hsia et al. 1994; Azechi et al. 2001).

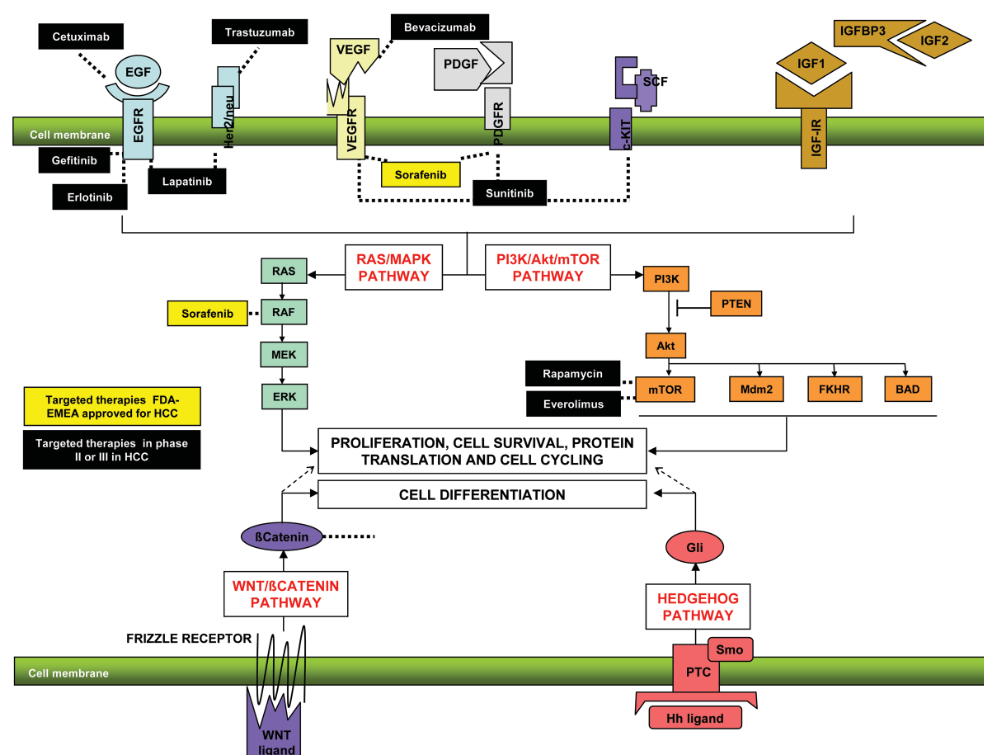


Figure 7. Dysregulated molecular mechanisms in HCC and therapies in HCC. From Llovet and Bruix, 2008.

p53 pathway is inactivated by a single point mutation in half of all tumors; in the other half, p53 is expressed at the normal levels but the signaling pathways that leads to cell cycle arrest and apoptosis are often defective (Giaccia and Kastan 1998). Several studies have reported p53 mutated in HCC. For instance, Aflatoxin B exposure in Africa and Asia is associated with p53 G-to-T mutation at the third position of codon 249 (Hsu et al. 1991; Hsu et al. 1993). Moreover, chronic infection with HBV and HCV viruses and exposure to oxidative stress, including hemochromatosis or inflammation, induce DNA damage and mutations in cancer-related genes, including TP53 (Hussain et al. 2007).

Evasion of apoptosis is due to deregulation of intrinsic or extrinsic apoptotic pathways. The majority of the HCCs show one or more alterations in the Fas pathway molecules, which inhibit Fas-mediated

apoptosis (Lee et al. 2001). TNF-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in various transformed cell lines but not in normal tissues. HCC cells constitutively express TRAIL, but different studies show that most HCC cells are resistant to TRAIL-mediated apoptosis (Chen et al. 2003); this resistance might be due to the blockage of TRAIL-R2/DR5 by the HBV core protein (Du et al. 2009), or through the overactivation of NF-κB and BCL-XL in HCC cells (Zender et al. 2005). Anti-apoptotic members of the BCL-2 family, such as MCL1 and BCL-XL are overexpressed in 50% of HCC and are associated with chemotherapeutic resistance (Takehara et al. 2001; Sieghart et al. 2006). Moreover, some pro-apoptotic members of the BH-3-only family, such as Bid, show decreased expression in HCC related to hepatitis B or C infection (Chen et al. 2001). Additionally, various members of the Inhibitor-of-apoptosis protein (IAP) family are overexpressed in HCC, XIAP, cIAP1 and survivin as it has been recently reviewed in Fabregat (2009).

Wnt/β-catenin. The signaling cascade is initiated extracellularly, when Wnt ligands stimulate the Frizzled receptors, which signal β-catenin to uncouple from E-cadherin and to translocate into the nucleus, where it regulates specific oncogenes, including c-myc, cyclin D, and survivin. Wnt/β-catenin signaling is significantly activated in HCC (Branda and Wands 2006). Furthermore, β-catenin mutations have been identified in hepatoblastoma (Giles et al. 2003). Interestingly, induction of EpCAM expression by Wnt/β-catenin signaling in HCC cell lines may be critical in maintaining hepatic cancer stem cell growth (Yamashita et al. 2007). As it has been reviewed, the Wnt pathway is involved in HCC arising from HBV/HCV infection and alcoholic liver cirrhosis, for instance HCV exposure can promote mutations of β-catenin (Aravalli et al. 2008). Moreover, in another review they summarize that the Wnt canonical pathways can also be activated by aberrant methylation of the tumor suppressors APC (adenomatous polyposis coli) and E-cadherin or by increase of autocrine/paracrine secretion of Wnt ligands (Llovet and Bruix 2008).

JAK/STAT Pathway. Signal transducers and activators of transcription (STATs) comprise a family of transcription factors that are activated by a variety of cytokines, hormones, and growth factors. Their activation occurs through tyrosine phosphorylation by Janus kinases (JAKs). There are three families of proteins that inhibit the STAT pathway, thereby preventing overactivation of cytokine-stimulated cells: the transcription of suppressors of cytokine signaling (SOCS) genes, the protein inhibitors of activated STATs (PIAS), and the SH2-containing proteins. JAK stimulation of STATs activates cell proliferation, migration, differentiation, and apoptosis; and deregulation of their inhibitors leads to human diseases, including cancer. Inactivation of SOCS-1 and SSI-1, a JAK-binding protein, and activation of the JAK/STAT pathway in HCC have been reported (Yoshikawa et al. 2001; Calvisi et al. 2006).

Involvement of MicroRNAs in Hepatocarcinogenesis. Several different classes of non-coding RNAs have been discovered in mammalian cells. These include small interfering RNAs, small nucleolar RNAs, and microRNAs (miRNAs). miRNAs measuring 20 to 23 nucleotides in length associate with the RNA-induced silencing complex (RISC) and interact with sites of imperfect complementarity in 30 untranslated regions (UTRs) of target mRNAs. Targeted transcripts subsequently undergo accelerated turnover and translational repression. Recent studies have demonstrated that alterations in microRNAs (miRNAs) genes lead to tumor formation, and several miRNAs which regulate either tumor suppression or promote tumor formation have been identified (Kent and Mendell 2006). Different reviews have highlighted the involvement of different miRNAs and HCC. For example, down-regulation of miR-15 and miR-16 results in overexpression of BCL-2, CDK6, and Cdc27, whereas overexpression of miR-21 causes suppression of PTEN and TPN1, whose overexpression has been found in HCC cell lines; the most abundant miRNA currently known in the liver is miR-122,

which is involved in cellular stress response, hepatocarcinogenesis, and inhibition of HCV replication and has been reported to target cyclin G1; additionally a miRNA profile obtained from microarray studies showed up-regulation of let-7a, miR- 21, miR-23, miR-130, miR-190, and miR-17-92 gene families in hepatomas (Aravalli et al. 2008; Minguez et al. 2009). Other studies have shown overexpression of miR-18, precursor miR-18 and miR-224 and decreased expression of miR-26a, miR-122, miR-199a*, miR-195, miR-199a, miR-200a and miR-125a in HCC compared with surrounding non-tumoral tissue (Murakami et al. 2006). Expression of miR-26a in liver cancer cells in vitro induces cell-cycle arrest associated with direct targeting of cyclins D2 and E2 (Kota et al. 2009).

Aberrant angiogenesis is a result of autocrine and paracrine secretion of VEGF (Vascular Endothelial Growt Factor), PDGF (Platelet-Derived Growth Factor), or angiopoietin- 2 or high-level of amplification of the VEGFA gene. VEGF is one of the most potent growth factors of the vascular endothelial cells. In a recent review on HCC, the VEGF expression was reported to associate not only with invasion and metastasis of HCC, but also with postoperative recurrence. Furthermore, angiopoietin-2/Tie2 signaling is another angiogenic pathway essential for HCC progression. VEGF and VEGFR pathways are required for the pathogenesis and progression of HCC (Tanaka and Arii 2009).

c-MET Signaling. Hepatocyte growth factor is a critical molecule for hepatocyte regeneration after injury (Fausto et al. 2006). Aberrant activity of MET has been described in human cancers as a result of MET amplification, germline or somatic mutations, transcriptional up-regulation, or HGF-dependent autocrine loops (Takami et al. 2007). Dysregulation of c-MET and HGF are common in HCC (Newell et al. 2008).

IGF Signaling. 16-40 % of human HCCs overexpress IGF-2 and the tumor suppressors IGF binding protein-1 (IGFBP-1), IGFBP-3, and IGFBP-4 are down-regulated. Several monoclonal antibodies and small molecules blocking IGF-1R are under early clinical investigations (A12, XL228). The interaction between IGF and EGF signaling may act as a mechanism of resistance for some tumors. In fact, the blockade of IGF-1R confers sensitivity to breast tumors resistant to trastuzumab, reviewed by Llovet and Bruix (2008).

PI3K/AKT/mTOR Pathway. PI3K (Phosphoinositide-3-Kinase) consists of p85 adaptor and p110 kinase subunits. After association with the intracellular domain of several RTK or specific substrates such as IRS-1, PI3K phosphorylates phosphatidylinositol 3,4,5-trisphosphate (PIP3) to generate phosphatidylinositol 4,5-bisphosphate (PIP2), which transduces phosphoinositide-dependent kinase (PDK), which in turn activates the serine-threonine kinase AKT. PIP3 is dephosphorylated by phosphatase and tensin homologue (PTEN), a tumor suppressor, which reverses this pathway. The activation of AKT leads to the phosphorylation and inactivation of several pro-apoptotic proteins and directs a number of downstream events that are responsible for cellular proliferation and apoptosis and is closely linked to the cell cycle (Luo et al. 2003). This pathway is known to be up-regulated in a subset of HCC patients: a recent review shows that on the one hand, AKT can be activated through a tyrosine kinase receptor (EGF or IGF signaling) or through constitutive activation of PI3K or loss of function of the tumor suppressor gene PTEN by epigenetic silencing or somatic mutations; on the other, they show that tumors with activated AKT have a worse prognosis (Llovet and Bruix 2008). Furthermore, mTOR is a downstream target of AKT, which acts as a central regulator of cell growth and proliferation, by sensing nutritional status and allowing progression from G1 to S phase, and plays a critical role in a subset of HCCs and its blockade with rapamycin or everolimus inhibits growth in HCC cell lines, and in experimental model (Villanueva et al. 2008).

RAS/Raf/ERK pathway. The intracellular mitogen-activated protein kinase (MAPK) family has five MAPK subgroups. These include the extracellular signal-regulated kinase protein homologs 1 and 2 (ERK1/2), big MAPK-1 (BMK-1/ERK5), c-Jun N-terminal kinase homologs 1, 2, and 3 (JNK1/2/3), stress-activated protein kinase 2 (SAPK-2) homologs α, β, and δ (p38 α/β/δ), and ERK6, also known as p38γ. The activity of these kinases is dependent upon dual phosphorylation of T and Y residues located in their activation loop. MAPKs are implicated in diverse cellular processes such as cell survival, differentiation, adhesion, and proliferation (Krishna and Narang 2008). Aberrant activation of the RAS/Raf-1/ERK pathway has been shown to be involved in the progression of HCC (Thomas 2009). However, the mechanism of dysregulation of ERK activation is poorly understood. Recently, Sprouty-related protein with an Ena/vasodilator-stimulated phosphoprotein homology-1 domain (Spred) was identified as a physiological inhibitor of the RAS/Raf-1/ERK pathway (Yoshida et al. 2006). In this study, they found that the expression levels of Spred-1 and -2 in human HCC tissues were frequently decreased. Moreover, the RAS pathway in HCC tumors is either hyperactivated due to mutations, downregulation of its inhibitors such as RASSF1A and NORE1A, or activated through Hepatitis B virus protein X (Tarn et al. 2001; Calvisi et al. 2006). Single point mutations in codon 13 of H-RAS, codon 12 of N-RAS, codon 61 of K-RAS (Challen et al. 1992) and codon 64 of K-RAS (Bai et al. 2003) were originally observed in HCC caused by various chemicals such as N-nitrosomorpholine, bleomycin, 1-nitropyrene, and methyl (acetoxymethyl) nitrosamine (Baba et al. 1997).

EGFR pathway. Epidermal growth factor (EGF) is a potent mitogen to hepatocytes. Unlike in other malignancies, the EGF receptor is rarely mutated in HCC, and several reports suggest an EGF-mediated autocrine growth stimulation of hepatoma cells (Yamaguchi et al. 1995; Hisaka et al. 1999). The EGFR/human EGFR1 (EGFR/HER1) and its ligands EGF and TGF-α have been shown to be highly involved in the development of hepatocarcinomas (Lee et al. 2007c); additionally several studies have supported the theory of an autocrine, paracrine, and endocrine mechanism of TGF-α and EGFR/HER1 on the proliferation of human HCC (Collier et al. 1993; Kira et al. 1997; Harada et al. 1999). As recently reviewed, multiple strategies to target EGFR signaling pathways have been developed; three tyrosine kinase inhibitors targeting EGFR have been tested in HCC: erlotinib and gefitinib targeting EGFR and lapatinib targeting both EGFR and HER2. Cetuximab, a monoclonal antibody against EGFR, has also been assessed (Fabregat et al. 2007; Newell et al. 2008; Zhu 2008; Tanaka and Arii 2009).

3. Apoptosis

Historically, two fundamentally different forms of cell death have been defined: apoptosis and necrosis. However, in the last years other forms of cell death have been proposed, such as caspase-independent cell death, autophagy, lysosomal membrane permeabilization (LMP) or the unfolded protein response (UPR) among others. Vastly, necrosis results in an early disruption of the cell membrane and in the progressive breakdown of ordered cell structures in response to violent environmental perturbations such as severe hypoxia/ischemia, extremes of temperature and mechanical trauma. In contrast, apoptosis or programmed cell death involves the activation of intracellular machinery in an energy dependent manner, which is tightly regulated and conserved throughout evolution (Vermeulen et al. 2005). Apoptosis is essential for the development and maintenance of tissue homeostasis. It allows the elimination of cells that are no longer needed. Indeed, an adult human produces and eliminates 60 billion cells daily, new cells are produced by division and old cells are eradicated by apoptosis. Apoptosis will also eliminate cells exposing the organism to danger. For example, virally infected cells or cells with damaged DNA will be removed by apoptosis. However, perturbations in apoptosis regulation contribute to numerous pathological conditions, including cancer, autoimmune and degenerative diseases (Giam et al. 2008; Dewson and Kluck 2009).

The morphological changes associated with apoptosis have been thoroughly reviewed, and are characterized by nuclear condensation, cell shrinkage, and loss of plasma membrane lipid asymmetry. Although more subtle, the Golgi, endoplasmic reticulum (ER) and mitochondrial networks also undergo pronounced fragmentation during apoptosis, finally plasma membrane blebbing occurs resulting in apoptotic bodies. Rapid engulfment of these through neighboring cells or macrophages prevents an inflammatory response as the one observed with necrotic cell death (Schattenberg et al. 2006; Taylor et al. 2008). In cell cultures, apoptotic bodies will lose the integrity of the plasma membrane during the late stages of apoptosis, followed by complete cell disintegration, also called secondary necrosis (Vermeulen et al. 2005).

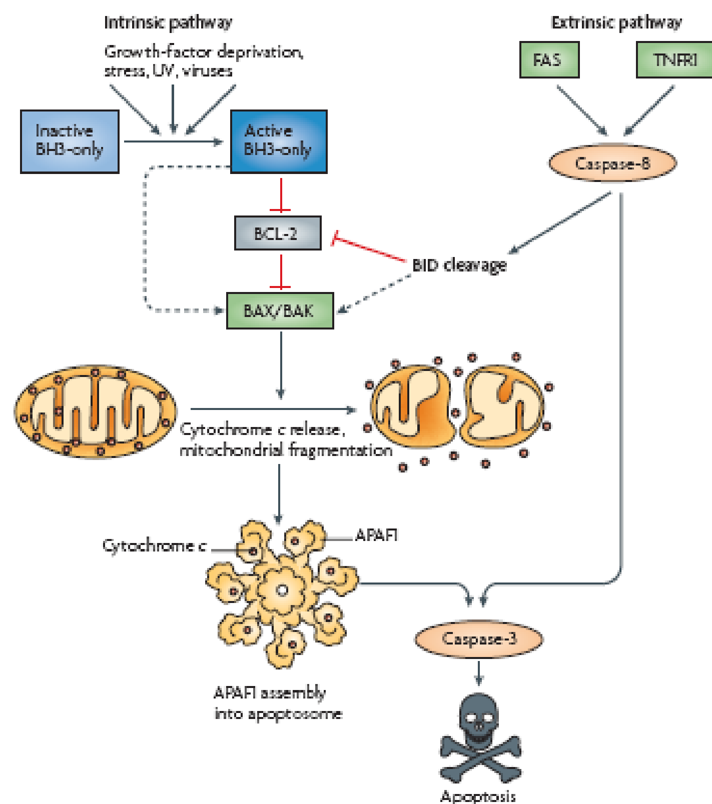


Figure 8. Apoptosis pathway, from Youle and Strasser (2008).

Apoptosis is an extensively reviewed topic; it occurs as the result of the activation of cysteine proteases called caspases, which dismantle the cells by degrading essential proteins. Two apoptotic

pathways converge on the activation of caspases: the ‘extrinsic pathway’, also called the ‘death receptor pathway’, involves the activation of cell-surface-expressed death receptors such as CD95 (also known as Fas receptor) or tumor necrosis factor receptor, and the activation of initiator caspases, 8 and 10. The ‘intrinsic pathway’, also called ‘mitochondrial’ or ‘BCL-2-regulated pathway’, is activated as a result of cellular stresses such as growth factor deprivation, DNA damage or exposure to cytotoxic substances. Mitochondria play a major role in the BCL-2-regulated pathway (Giam et al. 2008; Dewson and Kluck 2009). Both the mitochondrial pathway and the death receptor pathway are under suppression by a family of inhibitors of apoptosis (IAP). IAPs themselves are negatively regulated by IAP-binding proteins such as Smac/ Diablo, a mitochondrial protein that can be released in the cytosol during apoptosis induction. Omi/HtrA2 has also been described as an IAP-antagonist (Vermeulen et al. 2005; Youle and Strasser 2008).

3.1. Main protein families involved in apoptosis: Caspases, BCL-2 and IAPs.

3.1.1. Caspases

The name caspase is derived from Cys-dependent Asp specific protease, which means that the catalytic activity is governed by a conserved Cys side chain of the protease and by a stringent specificity for cleaving after Asp residues in substrates, which is very unusual for cellular proteases (Alnemri et al. 1996) . The Caspase Family has been broadly reviewed by several authors, the ‘‘initiator’’ caspases in mammals are caspase-1, -2, -4, -5, -8, -9, -10, -11, and -12, which respond to pro-apoptotic signals. The downstream ‘‘executioner’’ caspases in mammals are caspase-3, -6, -7, and -14. Caspases are synthesized as zymogens, their activation requires allosteric conformational changes and/or specific cleavage after a selective aspartate residue. Apoptosis ensues when the active caspases cleave their respective substrates, leading to the characteristic morphological features of apoptotic cell death. Each of the long-prodomain caspases contains in its prodomain a protein–protein interaction module, which allows it to bind to and associate with its upstream regulators. Caspase-8 and -10 contain a death-effector domain (DED), whereas caspase-2 and -9 contain a caspase activation and recruitment domain (CARD). Death adaptor modules usually mediate interactions through these domains, that is, DED/DED and CARD/CARD. Death adaptor modules might well act as integration platforms, binding to several different proteins, which could modulate their dimerization and hence caspase activation (Yi and Yuan 2009). Executioner caspases are constitutive dimers in the latent and the active forms; therefore, cleavage in the region of the intersubunit linker is the only requirement for activity for caspase-3 and caspase-7. Caspase-8, the initiator of the extrinsic apoptosis pathway, exists as an inactive monomeric form in the absence of a signal, like caspase-9, initiator of the intrinsic pathway. Both caspases are activated by dimerization, and need to be brought together to be activated *in vivo* by their respective activation platforms (Vermeulen et al. 2005; Riedl and Salvesen 2007). In mammals, four specific caspase activating complexes have been characterized. These complexes include the apoptosome, which mediates the activation of caspase-9 via interaction with the adaptor APAF-1 in the presence of cytochrome c; the death-inducing signaling complex (DISC), which mediates the activation of caspase-8 via interaction with the adaptor FADD; the inflammasomes, which mediate the activation of caspase-1 and caspase-5 via interaction with the adaptor apoptosis associated speck-like protein containing a CARD (ASC) or the family of NBD- and NOD-like receptors (NLRs); and the IDDosome, which mediates the activation of caspase-2 via interaction with the adaptors receptor-interacting protein- (RIP-) associated ICH-1/CED-3 homologous protein with a

death domain (RAIDD; also called CRADD) and p53-induced protein with a death domain (PIDD). The apoptosome is formed in response to cytochrome c release resulting from the loss of mitochondrial integrity, while the DISCs assemble after the stimulation of the death receptors by their respective ligands such as Fas ligand (FasL) or tumor necrosis factor-α (TNF-α) (Hengartner 2000; Yi and Yuan 2009).

Caspases selectively cleave a restricted set of target proteins, usually at one or at most a few positions. In most cases, caspases mediated proteolysis results in inactivation of the target protein. But caspases can also activate proteins, either directly, by cleaving off a negative regulatory domain, or indirectly, by inactivating a regulatory subunit. For instance, the caspase-activated DNase, or CAD, responsible for the degradation of genomic DNA into a ladder, pre-exists in living cells as an inactive complex with an inhibitory subunit, ICAD; activation of CAD occurs by means of caspase-3-mediated cleavage of the inhibitory subunit, resulting in the release and activation of the catalytic subunit. Nuclear fragmentation relies on the disintegration of the nuclear lamina and nuclear shrinking for which the proteolysis of lamins A, B and C by caspases is required. Loss of overall cell shape is probably caused by the cleavage of cytoskeletal proteins such as components of actin microfilaments, microtubular protein and intermediate filaments. Multiple transcription factors (such as AP-2α, BTF3, NFATc1 and NFATc2, NFκBp65 and SP1) are cleaved by caspases, as well as a range of translation initiation factors (including eIF2a, eIF3, eIF4B, eIF4E, eIF4G and eIF4H) and ribosomal proteins (for example, RPP0 and p70S6K) are also affected (Hengartner 2000; Vermeulen et al. 2005; Taylor et al. 2008). Finally, there is further evidence that caspases and their respective adaptor proteins mediate multiple cellular processes independent of apoptotic cell death per se. Non-apoptotic functions of caspases are involved in mediating immunity, cell fate specification, cell survival, cell cycle regulation, cell proliferation, and cell migration (Yi and Yuan 2009).

3.1.2. BCL-2 Family

BCL-2 family proteins regulate the intrinsic mitochondrial apoptotic pathway that is activated in response to a number of stress stimuli including growth-factor deprivation, cytokine-withdrawal, Ca^{++} flux or DNA-damage caused by UV or gamma-irradiation, but can also contribute to cell death triggered by death receptors (Frenzel et al. 2009). These proteins contain one or more BCL-2 homology (BH) domains, which share sequence homology and are important for heterodimeric interactions among members of the BCL-2 family (Yin et al. 1994; Chittenden et al. 1995). The family is divided in three main groups based on regions of BCL-2-homology (BH domains) and function. The first group is formed by anti-apoptotic proteins, which contain up to four BH domains. The BH1, 2 and 3 domains are critically involved in the creation of a hydrophobic groove where the α-helix formed by the BH3-domain of a pro-apoptotic partner binds to (Giam et al. 2008). Although it is known to be required for the anti-apoptotic function, BH4 domain molecular function still remains to be understood (Huang et

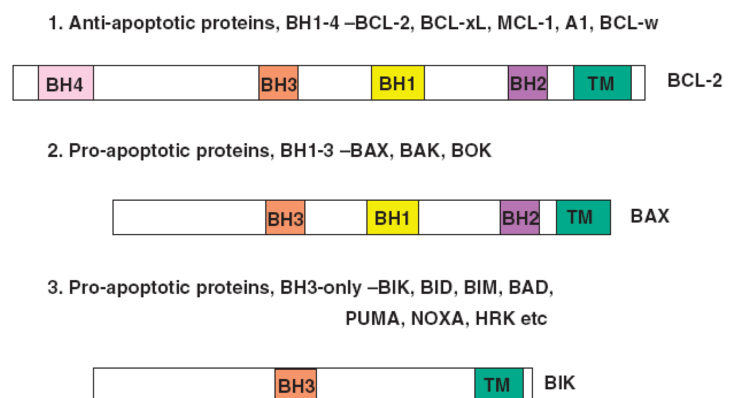


Figure 9. BCL-2 Family, From Yip and Reed, 2008.

al. 1998; Giam et al. 2008). The members of the anti-apoptotic group are BCL-2, BCL-XL, BCL-w, MCL1 and A1/BFL-1. The other two groups are pro-apoptotic, the first one posse's three BH (BH1-3) domains and is formed by BAX, BAK and BOK; the second group only contains the BH3-domain and is referred to as BH3-only proteins. The BH3 domain is an amphipathic α-helix that interacts with multidomain family members via the hydrophobic cleft formed by their BH1, BH2, and BH3 domains, this group is composed by BID, BIM/BOD, BAD, BIK/NBK, NOXA, PUMA/BBC3, BMF, HRK/DP5, EGl-1, BNIP3, BNIP3L and BECLIN (Certo et al. 2006; Lomonosova and Chinnadurai 2008). BH3-only proteins are pro-apoptotic and function as initial sensors of apoptotic signals that emanate from various cellular processes.

Using several BH3 peptides, the binding partners of each BH3-only protein has been elucidated. The molecular basis for this selectivity depends on critical amino acid residues in the amphipathic α-helical BH3 domain contacting key residues in the hydrophobic groove of the anti-apoptotic BCL-2-like pro-survival proteins (Liu et al. 2003; Pinon et al. 2008). For example, BAD and BMF selectively binds BCL-2, BCL-XL and BCL-w; NOXA specifically binds MCL1 and A1; and BIK and HRK peptides interacted more strongly with BCL-XL, BCL-w and A1. BIM, BID and PUMA can bind to all the anti-apoptotic proteins (Letai et al. 2002; Chen et al. 2005; Kuwana et al. 2005; Brunelle and Letai 2009). In addition, BIM, BID and PUMA were also found to interact with BAX (Marani et al. 2002; Cartron et al. 2004). Various cell death stimuli activate transcriptionally or post-transcriptionally one or more of BH3-only effectors that integrate and transmit the death signal through the multi-domain BH1–3 pro-apoptotic proteins, BAX and BAK (Lomonosova and Chinnadurai 2008). The BH1–3 pro-apoptotic proteins undergo conformational activation leading to oligomerization and insertion in the outer mitochondrial membrane; this process results in the permeabilization of outer mitochondrial membrane and the liberation of apoptogenic factors such as cytochrome c, which activate the caspase cascade leading to cellular death (Antonsson et al. 2000; Eskes et al. 2000; Korsmeyer et al. 2000; Wei et al. 2000).

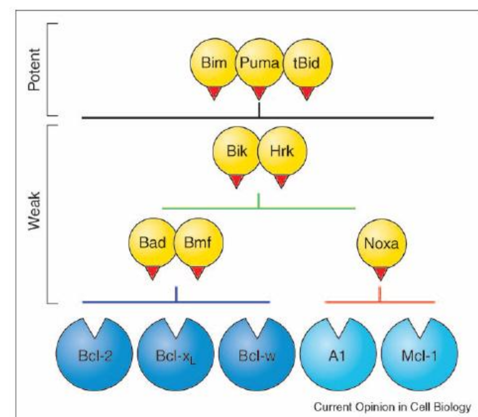


Figure 10. BH3-only partners, from Willis and Adams, 2005.

Under normal unstressed conditions, the level of many BH3-only proteins is very low or undetectable by common methods. Different ways of BH3 regulation are listed below. The transcription factor E2F-1 directly upregulates the expression of PUMA, NOXA, BIM and HRK/DP5 (Hershko and Ginsberg 2004) and BIK (Real et al. 2006). PUMA and NOXA are transcriptionally activated by p53 in response to DNA damage and other cell death signals (Oda et al. 2000; Nakano and Vousden 2001). The forkhead box transcription factor FOXO3a (FKHRL1) upregulates BIM expression (Dijkers et al. 2000; Gilley et al. 2003) and PUMA expression in response to cytokine/growth factors withdrawal (You et al. 2006). BCL-XL can be transcriptionally induced by growth factors through the JAK/STAT pathway to promote cell survival (Grad et al. 2000). Phosphorylation of BH3-only proteins has been reported to either positively or negatively influence their apoptotic activity. Phosphorylation of BAD abolishes its pro-apoptotic activity in response to survival factors (Zha et al. 1996). Members of the MAP kinase family, in particular JNK(c-Jun N-Terminal Kinase) and ERK, have opposing effects on BIM function upon phosphorylation. In response to growth factor-mediated activation of the RAS/RAF/ERK-signaling cascade, ERK mediated serine (Ser-59/69/77)

phosphorylation in human BIMEL (Ser-55/65/73 in mouse BIMEL) causes destabilization of the protein, facilitating ubiquitination and subsequent proteasomal degradation, thus promoting cell survival (Ley et al. 2005). In contrast, phosphorylation of threonine 112 in BIM by JNK increases its pro-apoptotic potential, by triggering release from the cytoskeleton (Lei and Davis 2003). BMF can be activated by release from actin–myosin motor complexes (Puthalakath et al. 2001). MCL1 is rapidly degraded by the ubiquitin–proteasome pathway in response to cytokine deprivation or other death stimuli (such as UV radiation) and can be up-regulated post-transcriptionally to prevent apoptosis by inhibiting its rate of degradation (Zhong et al. 2005). BIM and BMF protein expression can be induced by the addition of novel histone deacetylase inhibitors (HDACi) (Zhang et al. 2006a; Zhang et al. 2006b). Both BIM and BMF are targeted by TGF-β-mediated signals in mammary epithelial cells (Ramjaun et al. 2007). As a last example, BID is cleaved by caspase-8 resulting in an N-terminally truncated form (tBID) (Li et al. 1998b). tBID targets to mitochondria and amplifies death receptor-induced cell death through the mitochondrial pathway.

3.1.3. IAPs

Inhibitors of apoptosis proteins (IAPs) are a conserved family of proteins identified in species ranging from virus, yeasts, nematodes, fishes, flies and mammals. The IAPs effectively suppress apoptosis induced by a variety of stimuli, including death receptor activation, growth factor withdrawal, ionizing radiation, viral infection, and genotoxic damage. The common structural feature is the presence of at least one Baculovirus IAP Repeat (BIR) domain. IAPs contain 1–3 BIR domains of 70–80 amino acids encoding a C2HC-type zinc-finger motif. Hence, IAPs are also known as BIR-containing proteins (BIRCs). Eight members of this family have been described in humans: BIRC1/NAIP, BIRC2/cIAP1, BIRC3/cIAP2, BIRC4/XIAP, BIRC5/Survivin, BIRC6/Apollon/BRUCE, BIRC7/ML-IAP and BIRC8/ILP2; interestingly, most of them display anti-apoptotic properties when overexpressed reviewed by Hunter et al. (2007).

IAPs are composed by several well conserved protein domains. The first one is the BIR domain that defines IAP family (Sun et al. 1999). The second most frequent conserved protein domain is the RING (C-terminal Ring zinc-finger) domain that behaves as an E3 ubiquitin ligase (Vaux and Silke 2005a; Vaux and Silke 2005b). Thanks to the presence of the RING domain, IAPs mediate, at least in vitro, their own ubiquitylation, or ubiquitylation of binding partners such as caspase-3, -7 and -9, TRAF1 and 2, Smac/Diablo, NEMO/IKKγ (IκB kinase γ) and ASK1 (apoptotic signal-regulating kinase 1); IAP-induced ubiquitylation induces proteasomal degradation of their targets, reviewed by Dubrez-Daloz et al. (2008). In addition to BIRs and RING, BIRC2/cIAP1 and BIRC3/cIAP2 also present a central CARD. The function of CARD in BIRC2/cIAP1 and 2 is still unknown; probably it is related to protein-protein interaction (Dubrez-Daloz et al. 2008; LaCasse et al. 2008). BIRC4/XIAP is a direct inhibitor of proteolytic activity of the initiator caspase-9 and executors caspase-3 and -7 through two-site binding mechanism, one of them being the IAP-binding motif (IBM) (Scott et al. 2005). Moreover, BIRC4/XIAP has been shown to be able to ubiquitylate the active form of caspase-3 (Suzuki et al. 2001) and -9 (Morizane et al. 2005) and target them for degradation to the proteasome-complex. Even though BIRC2/cIAP1, BIRC3/cIAP2 and BIRC1/NAIP can bind caspases through the IBM-mediated interaction, they are not direct caspase inhibitors (Eckelman et al. 2006), they probably exert their anti-apoptotic function by ubiquitylation.

IAPs are negatively regulated by different proteins, as summarized in recent reviews, the most known are SMAC (also known as DIABLO) and Omi (also known as HtrA2). When a cell undergoes

apoptosis these molecules are released from the intermembrane space into the cytosol, where they bind cIAP1 and -2 via the IBM domain. SMAC presents several isoforms; following an apoptotic stress, Smac3 and Smac are released from the mitochondria into the cytosol where it interacts with the BIR2 and BIR3 domains of XIAP. Smac3 isoform can also induce the acceleration of XIAP auto-ubiquitination and destruction, whereas Smac only seems to have this effect on c-IAP1 and c-IAP2. Omi exerts its pro-apoptotic activity both by disrupting caspase-IAP interaction, as well as by its serine protease activity (Hunter et al. 2007; Srinivasula and Ashwell 2008).

Recently, new evidence has shown the importance of IAPs in cell proliferation, differentiation, signaling, motility and in immune system. Survivin is transcriptionally restricted to expression during the G2/M phase of the cell cycle where it is proposed to function as a mitotic spindle checkpoint protein. Survivin appears to associate with caspase-3 during mitosis and seems to suppress caspase-mediated cleavage of centrosome-associated p21Waf1 (Li et al. 1998a; Li et al. 1999). Moreover, IAPs can interact with other signaling pathways, it has been demonstrated that overexpression of XIAP BIR1 interacts with TAB1 (TGF-β activating kinase 1 (TAK1) associating subunit 1), which could trigger XIAP-induced MAPKKK TAK1 activation and TGF-β signaling (Lu et al. 2007). Overexpression of BIRC4/XIAP can also activate the transcription of TGF-β, NF-κB and JNK-responsive genes, in a Smad4 dependent way (Birkey Reffey et al. 2001). Both c-IAP1 and -2 interact with TRAF1 and 2 via their BIR1 regions which greatly enhances TRAF2-induced NF-κB activity (Samuel et al. 2006; Srinivasula and Ashwell 2008).

3.2. Extrinsic pathway

The extrinsic pathway, which has been the focus of many reviews, is initiated after binding of trimeric ligand molecules to corresponding preassembled receptor multimers. The extracellular ligands are members of the tumor necrosis factor (TNF) ligand superfamily CD95L, TNF and TRAIL. These cytokines induce their physiological function through their cognate receptors, the CD95 receptor (Apo1/Fas receptor), TNF receptor type 1 (TNF-R1, p55/65, CD120a) and type 2 (TNF-R2, p75/80, CD120b), TRAIL receptor type 1 and type 2. Ligand–receptor interactions occur at the plasma membrane and results in conformational changes of the receptor, initiating the assembly of an early intracellular signaling complex, to which downstream signaling molecules are subsequently recruited. The intracellular domains of death receptors do not present an intrinsic kinase activity and depend on homophilic protein–protein interactions for the initiation of cell signaling. The apoptotic cell death pathway is activated following recruitment of the Fas-associated death domain (FADD/MORT1) that contains a death domain (DD) that interacts with the DD present in Fas receptor formin a death receptor-induced signaling complex (DISC). Besides a DD, FADD contains a death effector domain (DED) which recruits the DED-containing pro-caspase-8 into the DISC. Pro-caspase-8 will be recruited and activated. Caspase-8 activation leads to the activation of the effector caspase-3 which carries out the cleavage of different substrates ultimately resulting in apoptosis (Vermeulen et al. 2005; Schattenberg et al. 2006; Riedl and Salvesen 2007). In the case of TNF, the adaptor protein is TRADD, which apart from inducing apoptosis it is also able to induce survival signaling. To do so, TRADD recruits the adaptor molecule TNF receptor-associated factor-2 (TRAF2), which in turn recruits c-IAP1 and c-IAP2. The binding of the receptor interaction protein (RIP), the third protein able to interact with TRADD, leads to activation of the transcription factor NF-κB, resulting in transcription of anti-apoptotic genes and promoting cell survival (Vermeulen et al. 2005).

Usually the extrinsic apoptotic pathway activates caspases without the participation of mitochondria. However, in certain cell types, the extrinsic pathway also induces mitochondrial damage by cleaving the pro-apoptotic BCL-2 family protein BID to its activated truncated form (tBID), which leads to BAK and BAX activation resulting in a molecular link between both apoptotic pathways (Korsmeyer et al. 2000; Dewson and Kluck 2009).

3.3. Intrinsic pathway

The intrinsic apoptotic pathway is initiated in response to a variety of stress signals (Willis and Adams 2005), and a complex interplay of BCL-2 proteins transduces this signal to the mitochondrial outer membrane (OM) to initiate BAK and BAX activation, oligomerization and OM damage. BAK- or BAX-mediated mitochondrial outer membrane permeabilization (MOMP) is recognized as the point of no return in mammalian apoptosis. BAK and BAX double-knockout cells fail to undergo MOMP in response to many different death stimuli (Wei et al. 2001; Kuwana et al. 2005). BAX proteins can be found as monomers in the cytosol or loosely associated with the outer mitochondrial membrane when not activated. BAX translocates to and inserts into the mitochondrial outer membrane during the activation process (Billen et al. 2008). BAK is inserted into the outer mitochondrial membrane even when not activated (Wei et al. 2000). BAX and BAK oligomers form pores in and cause permeabilization of the outer mitochondrial membrane. MOMP releases numerous pro-apoptotic proteins into the cytosol which participate in caspase activation (for example, cytochrome c) and that neutralize endogenous inhibitors of caspases (for example, SMAC; OMI/Htra2), as well as releasing several mediators of caspase-independent necrotic cell death (Galluzzi et al. 2008; Yip and Reed 2008). MOMP also releases several proteins that contribute to non-apoptotic cell death, including DNase, endonuclease G and AIF, flavoproteins reported to enter the nucleus and promote genome destruction (Penninger and Kroemer 2003). The major weapon in the mitochondria is cytochrome c, which, when introduced into the cytosol, binds and activates APAF-1. In the presence of cytochrome c and ATP, the CARD domain of APAF-1 binds with the CARD domain of procaspase-9, and this forms the apoptosome, which promotes activation of pro-caspase-9. Caspase-9 will in turn activate downstream caspases like caspase-3, caspase-6 and caspase-7 (Riedl and Salvesen 2007). Activated caspases rapidly cleave multiple substrates which result in cell death. Notably, these caspases act downstream of the point of no return and, therefore, blocking their activation delays cell death but does not prevent eventual cell death (Ekert et al. 2004). In addition, uncoupling of mitochondrial oxidative phosphorylation is observed during apoptosis, resulting in the loss of mitochondrial transmembrane potential (Green and Reed 1998).

Two main models of activation of BAX and BAK have been proposed, they are known as the ‘indirect’ or ‘direct’ models. According to the indirect model, BAX and BAK must be sequestered by anti-apoptotic proteins of the BCL-2 family to prevent their activation, upon a death stimuli BH3-only proteins are activated and bind the anti-apoptotic proteins in order to liberate BAX and BAK, allowing their activation (Chen et al. 2005; Willis et al. 2005). In this model a cell undergoes apoptosis when all pro-survival BCL-2 molecules expressed in a cell are neutralized by BH3-only proteins. The direct model divides the BH3-only proteins in two groups the “activator” proteins (BIM, tBID and PUMA) which apart from binding to all anti-apoptotic BCL-2 family members can also directly induce oligomerization of BAK and BAX (Letai et al. 2002; Kuwana et al. 2005), the remaining BH3-only proteins are called “sensitizers” or “de-repressors” and they cannot interact directly with the multidomain pro-apoptotic proteins. According to the direct model, anti-apoptotic proteins prevent death by binding and sequestering activator BH3-only proteins, and also by binding

any monomeric and activated BAX or BAK protein. Upon apoptotic stimuli, sensitizer BH3-only proteins bind to the anti-apoptotic proteins causing the release of activator BH3-only proteins, that subsequently will induce BAX and BAK activation (Cartron et al. 2004; Certo et al. 2006). A recent report (Merino et al. 2009) has shown that binding to anti-apoptotic BCL-2 family proteins does not account for BIM's pro-apoptotic activity as it is proposed by the indirect model, and that total BIM activity requires its ability to directly interact with multidomain pro-apoptotic proteins BAX and BAK. Therefore, they proposed that initiation of apoptosis requires features of both direct and indirect model.

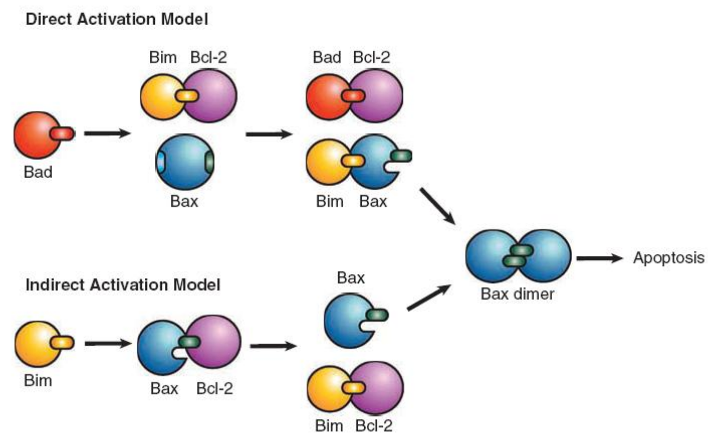


Figure 11. Models of BCL-2 avtivation, From Giam et al. 2009.

It is possible that more BH3-only proteins with an activator function may exist. Other proteins capable of triggering mitochondrial damage have been reported. Some, like BCL2L12 (Scorilas et al. 2001), belong to the BCL-2 family, whereas others, such as p53 (Chipuk et al. 2004) do not contain any BH domain. Calpain-mediated cleavage of Atg5 (an autophagy effector) resulted in the translocation of truncated Atg5 from the cytosol to mitochondria, association with BCL-XL and apoptotic cell death (Yousefi et al. 2006).

3.4. Other types of cell death

In this section, I briefly mention other types of cell death that have been extensively reviewed. Accumulation of misfolded proteins in the endoplasmic reticulum (ER) activates the unfolded protein response (UPR). In the ER, proteins obtain their mature conformation after proper post-translational modification, folding and oligomerization; when there is an excessive number of proteins for the ER capacity it may result in the accumulation of misfolded proteins in the ER, leading to repair of ER folding or, in case of severe damage, to initiation of apoptosis (Rasheva and Domingos 2009). In the lumen of the ER, Ca^{2+} is stored, and disturbance of the Ca^{2+} homeostasis initiates apoptosis (Jaattela 2004). Mitochondrial involvement in ER stress-induced cell death has been shown by the release of cytochrome c from mitochondria after induction of ER stress, for which BAX activation is required (Scorrano et al. 2003). In addition, BCL-2 has been shown to inhibit ER stress-induced apoptosis (McCullough et al. 2001). Caspase-12 is localized on the ER membrane and is specifically activated during ER stress-induced apoptosis, its mechanism of activation is unclear, and it involves calpain dependent removal of the pro-domain and self-cleavage (Nakagawa and Yuan 2000; Nakagawa et al. 2000). Calpains are Ca^{2+}-dependent cytosolic cysteine proteases which can also mediate caspase-independent apoptosis (Vermeulen et al. 2005).

Necrosis has been defined as a type of cell death that lacks the features of apoptosis and autophagy, and is often considered to be an uncontrolled process. However, recent research suggests that its course might be regulated. Following a signaling- or damage-induced lesion, necrosis can include

signs of controlled processes such as mitochondrial dysfunction, enhanced generation of reactive oxygen species, ATP depletion, proteolysis by calpains and cathepsins, and early plasma membrane rupture. In addition, the inhibition of specific proteins involved in regulating apoptosis or autophagy can change the morphological appearance of cell death to necrosis, reviewed in Turk and Turk (2009).

Prolonged nutrient deprivation invokes autophagy, an evolutionarily conserved response for catabolizing macromolecules and organelles, thereby generating substrates for ATP production (Yip and Reed 2008). Autophagy is a lysosomal degradative, initially described as a type of cellular death (Kroemer and Jaattela 2005). Nowadays, it remains unclear whether autophagy represents an independent mode of programmed cell death, a mechanism of cell death when apoptosis is inhibited or simply a stress response that is activated in damaged cells (Mizushima 2007). Indeed, it has been proposed that the frequent presence of autophagosomes in dying cells is due to an active autophagy process as an attempt to eliminate toxic molecules or damaged organelles, if the autophagic system of defense is overwhelmed then cells die from apoptosis or necrosis (Boya and Kroemer 2008). Autophagy begins when cellular membrane surrounds cytoplasmic organelles and/or a portion of the cytosol, forming a closed double membrane vacuole, the autophagosome, containing cytoplasmic material destined for degradation. Autophagosomes mature in a stepwise process that involves fusion events with endosomal and/or lysosomal vesicles that generate amphisomes or autolysosomes, respectively (Kroemer and Jaattela 2005). Autophagy might prevent cell death under some conditions. However, when apoptosis is inhibited, high levels of autophagy can function as a cell death effector mechanism**.** Autophagy is regulated by the BCL-2 family; BCL-2 and BCL-XL suppress autophagy by binding the protein BECLIN 1 (Atg6) (Pattingre et al. 2005), an essential component of the mammalian autophagy system that marks autophagic vesicles for fusion with lysosomes. BNIP3, BAD, EGL1, NOXA, PUMA, BIM and BIK competitively disrupt the interaction between the anti-apoptotic members of the family and BECLIN (Galluzzi et al. 2008; Yip and Reed 2008).

Lysosomal membrane permeabilization (LMP) is another mechanism for the induction of cell death. Complete disruption of lysosomes provokes uncontrolled cell death by necrosis. In contrast, partial and selective LMP induces the controlled dismantling of the cell by apoptosis. Lysosomal proteases that have been implicated in cell death are those cathepsins that remain active at neutral pH, such as cathepsin B (CB), cathepsin D (CD) and cathepsin L (CL). These proteases activate apoptotic effectors such as mitochondria and/or caspases. The distinctive sign of LMP is the translocation of soluble lysosomal components (including enzymes) from the lysosomal lumen to the cytosol. LMP can be induced by classic apoptotic stimuli, intracellular second messengers such as reactive oxygen species and sphingosine, as well as by lysosomotropic toxins, death receptors and p53 (Conus and Simon 2008). It has also been observed that BIM and BAX can translocate from the cytosol to the lysosomal membrane and induce LMP, these events occur upstream of MOMP (Feldstein et al. 2004; Boya and Kroemer 2008). Lysosomal enzymes (CB and CD) may directly attack lysosomes and facilitate LMP-mediated cell death. In addition, other proteases like calpains can induce lysosomal destabilization and LMP-dependent cell death (Cheung et al. 2007). Often, LMP causes mitochondrial outer-membrane permeabilization and caspase activation. One of the links between LMP and MOMP is BID, which can be cleaved by several cathepsins (Cirman et al. 2004). Several reports indicate that LMP can initiate a caspase-independent cell death pathway. AIF has been shown to be one of the main effectors of caspase-independent cell death. During apoptosis, AIF translocates from mitochondria to the nucleus where it induces DNA degradation. AIF may be responsible for promoting caspase-independent cell death after LMP (Modjtahedi et al. 2006).

4. ROS and NOX

4.1. Reactive Oxygen Species

Reactive oxygen species (ROS) are a group of molecules produced in cells when oxygen is metabolized. This group includes superoxide anion ($O_2\cdot-$), nitric oxide (NO·), hydrogen peroxide (H_2O_2), and the hydroxyl anion (OH·). ROS, and $O_2\cdot-$ in particular, are produced by a variety of enzymes, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox), xanthine oxidase, lipoxygenase, cyclooxygenases and myeloperoxidase. ROS generation has also been identified as a by-product in a variety of physiological processes including cytochrome P-450 oxidase uncoupling, endothelial nitric oxide synthase (eNOS) uncoupling, mitochondrial respiration, and activation of various peroxisome oxidases. If the concentration of H_2O_2 is high, these oxidation processes may lead to irreversible damage, followed by cell death. However, this is not always the case, and H_2O_2 at low concentrations is capable of reversible inhibition of many enzymes, including phosphatases, and to modulate intracellular signaling (Brown and Griendling 2009; Groeger et al. 2009). ROS generation is generally a cascade of reactions that starts with the production of superoxide. Two molecules of superoxide can react to generate hydrogen peroxide (H_2O_2) in a reaction known as dismutation, which is accelerated by the enzyme superoxide dismutase (SOD). In the presence of iron, superoxide and H_2O_2 react to generate hydroxyl radicals. Other elements in the cascade of ROS generation include the reaction of superoxide with nitric oxide (NO) to form peroxynitrite (OONO−); the peroxidase-catalyzed formation of hypochlorous acid (HOCl), from hydrogen peroxide and chloride by the phagocyte enzyme myeloperoxidase (MPO); singlet oxygen, which might be formed from oxygen in areas of inflammation; ozone, which can be generated from singlet oxygen by antibody molecules; and the iron-catalyzed Fenton reaction (Lambeth 2004; Bedard and Krause 2007). Moreover, the formation of OONO− from $O_2\cdot-$ can then lead to reversible glutathionylation of proteins on reactive cysteines. Finally, both $O_2\cdot-$ and H_2O_2 react with protein thiols, but due to the greater stability and diffusibility of H_2O_2, it is most likely that H_2O_2 is responsible of reacting with the protein thiols involved in ROS signaling (Brown and Griendling 2009).

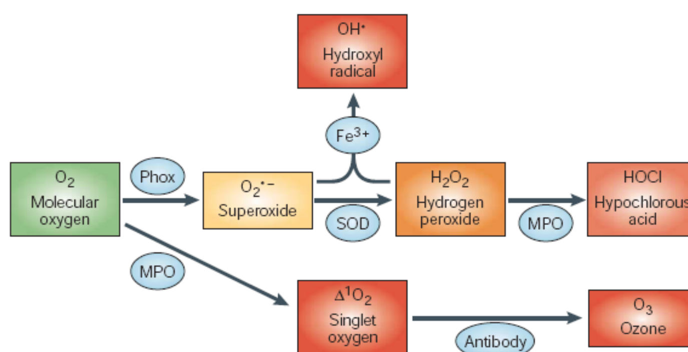


Figure 12. ROS transformation, from Lambet 2004.

Glutathione (GSH) is a tripeptide made of glutamate, cysteine (Cys), and glycine which is an ubiquitous intracellular antioxidant important for cellular protection against ROS, electrophiles, and xenobiotics. With regard to proteins, the formation of a mixed GSH-protein disulfide (glutathionylation) has been shown to protect proteins against irreversible oxidation. The intracellular space typically contains GSH in the range of 3 to 10 mM, GSH will attenuate, but not abrogate, the reactivity of H_2O_2. Hydrogen peroxide is more stable than $O_2\cdot-$ and is also capable of crossing biological membranes. H_2O_2 is also tightly regulated biologically by catalase, glutathione peroxidase, and peroxiredoxins. In mammals, three isoforms of superoxide dismutase are known: cytoplasmic SOD, which is a copper/zinc dismutase (SOD1); mitochondrial manganese SOD (SOD2); and extracellular Cu/Zn SOD (SOD3; ec-SOD). As mentioned before, the primary function of the SODs is

to catalyze the dismutation of superoxide to hydrogen peroxide, which can then either function in signaling reactions or be further reduced to water by catalase or a peroxidase. The glutathione peroxidases (GPxs) reduce peroxides by transferring electrons from GSH with the generation of oxidized glutathione (GSSG). Catalase efficiently converts H_2O_2 to water and oxygen. However, it is likely that the main intracellular H_2O_2 scavengers are the peroxiredoxins. Peroxiredoxins remove H_2O_2 to yield water and form an intermolecular disulfide bond, which then can be reduced by thioredoxin (Trx). H_2O_2 can reversibly react with cysteine residues on proteins to initially form a disulfide bond (–SSR) and sulfenic acid (–SOH). Sulfinic acid (–SO2H) and sulfonic acid (–SO3H) can be formed by additional oxidation; however, these latter reactions are essentially irreversible, and not useful for signaling (Brown and Griendling 2009; Chen et al. 2009).

ROS react non-specifically and rapidly with biomolecules, including DNA, proteins, lipids and carbohydrates, and various investigations have elucidated roles for ROS in causing molecular damage such as DNA mutations, lipid peroxidation and protein oxidations. Thus, ROS have historically been viewed as a harmful but unavoidable consequence of an aerobic lifestyle. However, in the last years it has been observed that ROS are also generated in a regulated manner by NADPH oxidases proteins in response to growth factors and cytokines. This observation has been accompanied by various studies that have shown that ROS also play diverse roles in biology, including host defense, hormone biosynthesis, fertilization, and redox signaling involved in mitogenesis, apoptosis, migration, or oxygen sensing (Geiszt and Leto 2004; Lambeth 2004).

4.2. NADPH Oxidase Family

NADPH oxidase (Nox) proteins are membrane-associated, multiunit enzymes that catalyze the reduction of oxygen using NADPH as an electron donor. Nox proteins produce superoxide (O2·–) via a single electron reduction. The electron travels from NADPH down an electrochemical gradient first to flavin adenine dinucleotide (FAD), then through the Nox heme groups, and finally across the membrane to oxygen, forming O2·– (Brown and Griendling 2009). Nox2 is the prototype member of this family and was originally called gp91phox (phagocytic oxidase). It is highly expressed in neutrophils and macrophages, in which it produces an oxidative burst to destroy pathogens. This oxidase is inactive in resting neutrophils, but it is activated by exposure to microorganisms or inflammatory mediators, resulting in non-mitochondrial production of ROS. In 1999, the first of the NOX homologues of gp91phox was described, NOX1. Subsequently, the others members of this family were identified and cloned (Lambeth 2004; Groeger et al. 2009).

The mammalian Nox enzymes can be classified into three groups, based on the presence of domains in addition to the Nox flavocytochrome domain that consists of six transmembrane helices with binding sites for NADPH and FAD, and heme-coordinating histidine residues. Nox1, Nox2, Nox3, and Nox4 all contain the flavocytochrome catalytic moiety. Nox5 is the single member of the second group which has, in addition to the basic Nox catalytic moiety, an amino-terminal calmodulin-like domain that contains four calcium-binding EF-hand structures, which confer its dependence on calcium for activation. A third group of Nox's are the "dual oxidases" or Duox (Duox 1 and 2), which contain peroxidase homology domains, which means that these two members of the family produce H_2O_2 and not O_2·– as their final product; they also have two EF domains and so their activation is also calcium dependent. Nox1, - 2, - 3, and - 4 are similar in sequence. Their amino acid homology ranges from 56% (Nox1 and Nox2), and 58% (Nox2 and Nox3), to 39% (Nox2 and Nox4). Nox5 is

significantly different because of its four EF domains (Lambeth et al. 2007; Chen et al. 2009; Groeger et al. 2009).

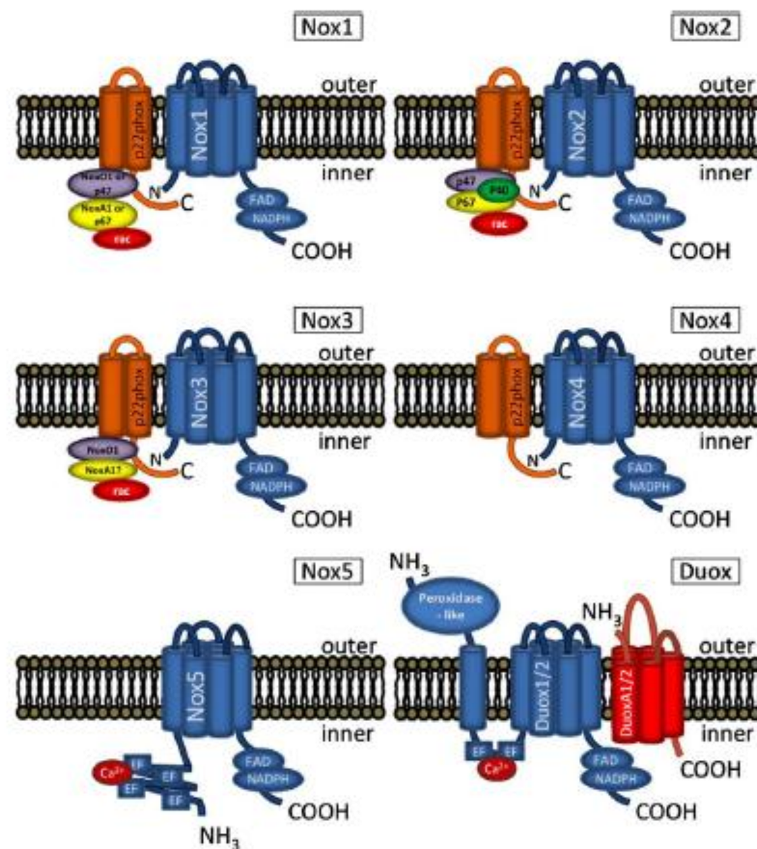


Figure 13. NOX Family, From Brown and Griendling, 2009.

The phagocytic NADPH oxidase is a multi-subunit enzyme complex with both membrane and cytosolic components. The membrane subunits gp91phox and p22phox make up the flavocytochrome b558 component of the phagocytic Nox. The cytosolic subunits include p47phox, p67phox, p40phox, and the small GTPase Rac1/2. In the resting state the cytosolic components remain quiescent in the cytoplasm, and the membrane-bound cytochrome b558 complex is inactive. Upon stimulation, the cytosolic subunits are translocated to the membrane to bind the cytochrome b558 components, leading to activation of the NADPH oxidase complex. Included in this activation process are the phosphorylation of p47phox and p67phox, and the conversion of GDP-bound Rac1/2 into GTP-bound forms through the activation of a Rac guanine nucleotide exchange factor. Other NADPH oxidases share several of the coactivator subunits with Nox2, but can also use p47 and p67 homologues, known as Noxo1 (Nox organizer protein 1) and Noxa1 (Nox activator protein 1) (Lambeth et al. 2007; Chen et al. 2009; Oakley et al. 2009). All Nox isoforms appear to have a mutual stabilizing relationship with the p22phox subunit (except for Nox5). The other function described for p22phox is binding the organizer subunits, this function is relevant for Nox1-3, but not for Nox4 (Bedard and Krause 2007; Chan et al. 2009).

Nox genes only exist in eukaryotes but could not be found in prokaryotes. The Nox family of genes emerged early in eukaryote evolution (Chan et al. 2009). Expression of NADPH oxidases is ubiquitous in mammals, though the individual Nox isoforms have different distributions between tissues and species. Nox proteins have been shown to regulate many fundamental physiological processes, including cell growth, differentiation, apoptosis, and cytoskeletal remodeling. The roles of different Nox family members, though they all produce $O_2{\cdot}-$, are distinct. This is due in part to compartmentalization within the cell. One of the main modes of action of NADPH oxidase derived ROS is by modulating different kinase activities. A well recognized intracellular target of NADPH oxidase-derived ROS is PTP, a large family of enzymes that dephosphorylate tyrosine residues. All PTPs contain an essential cysteine residue in the active site. This cysteine residue is highly susceptible

to oxidation by ROS, leading to reversible inhibition of the enzymatic activity (Brown and Griendling 2009; Chan et al. 2009; Chen et al. 2009; Groeger et al. 2009). Activities of many PTP members, including PTP1B (Protein Tyrosine Phosphatase1B) (Sharma et al. 2008), SHP-1 (Lee and Esselman 2002) and -2 (Tabet et al. 2008), PTPα (Blanchetot et al. 2002), CD45 (Lee and Esselman 2002) and low-molecular-weight PTP (LMW-PTP) (Lee et al. 2007a), are known to be affected by ROS, and subsequently it leads to the activation of AKT, JAK2 among other kinases. In addition, NADPH oxidase and subsequent ROS production facilitates RAS activity through glutathionylation of Cys118, leading to ERK activation (Adachi et al. 2004). Finally, there are different reports which have shown that ROS oxidized SRC on cysteine residues inducing SRC activity, which allows the specific ligand-independent phosphorylation and activation of EGFR, downstream of which ERK and AKT signaling pathways are activated (Callera et al. 2005; Peshavariya et al. 2009). Hepatocytes contain a number of systems that might be involved in the generation of ROS. In addition to Nox family NADPH oxidases, there are other sources of ROS in hepatocytes such as mitochondria, cytochrome *P*-450 enzymes, ER oxidoreductases, and cytosolic peroxisomal and xanthine oxidases (Bedard and Krause 2007). Finally, hepatocytes generate ROS in response to a wide variety of endogenous and exogenous stimuli, including CD95 (Fas) ligand (Reinehr et al. 2005), and alcohol (Bailey and Cunningham 1998). In response to TGF-β, fetal rat hepatocytes induce the activity of Nox1 and Nox4, resulting in two opposite outcomes. On the one hand, TGF-β-induced apoptosis requires the up-regulation of Nox4 expression and ROS production, since Nox4 silencing by siRNA targeted knock-down or blockage of ROS production results in the impairment of apoptosis (Herrera et al. 2001a; Carmona-Cuenca et al. 2008). On the other hand, TGF-β increases the levels of Rac1 protein, which is a regulator of both Nox1 and Nox2, in fetal rat hepatocytes; TGF-β-induced expression of EGF-like ligands (TGF-α and HB-EGF) is mediated by the activation of NF-κB through a ROS dependent manner (Murillo et al. 2007). Additionally, in FaO rat hepatoma cells, TGF-β induces the expression of Nox1, where it plays a pro-survival role, as its silencing by specific siRNA targeted knock-down results in an increased activation of caspase-3 by TGF-β (Sancho et al. 2009).

The most commonly used NOX inhibitor is the iodonium- derivative diphenylene iodonium (DPI). DPI is a nonspecific inhibitor of many different electron transporters. It inhibits not only all of the NOX isoforms, but also nitric oxide synthase, xanthine oxidase, mitochondrial complex I, and cytochrome *P*-450 reductase (Bedard and Krause 2007).

4.2.1. NOX4

Nox4 is highly expressed in the kidney, but has been found to be expressed in other cell types including mesangial cells, smooth muscle cells, endothelial cells, fibroblasts, keratinocytes, osteoclasts, neurons, and hepatocytes (Bedard and Krause 2007; Lambeth et al. 2007; Brown and Griendling 2009). Nox4, originally Renox, is unique among the Nox family in that it only requires the membrane subunit p22phox for ROS-producing activity, and appears to be constitutively active (Krause 2004). It has been proposed that Nox4 is an inducible Nox, and its activity is proportional to Nox4 protein expression alone. Recently, a novel p22phox binding partner has been described, Poldip2, which associates with p22phox, Nox1, and Nox4 and colocalizes with p22phox at sites of Nox4 localization. Poldip2 increases Nox4 enzymatic activity and positively regulates basal reactive oxygen species production in Vascular smooth muscle cells (VSMCs) (Lyle et al. 2009). Nox4 localization has been shown to be in focal adhesions (Hilenski et al. 2004), the nucleus (Kuroda et al. 2005), in the perinuclear space (Mittal et al. 2007), the endoplasmic reticulum (Ambasta et al. 2004),

and the mitochondria (Block et al. 2009), the complex NOX4-p22 has also been observed in the cytosol (Weyemi et al. 2009).

Nox4-derived ROS have been implicated in a variety of physiological processes, including cellular senescence, apoptosis, survival, insulin signaling, migration, endoplasmatic reticulum stress, and differentiation (Brown and Griendling 2009). Nox4 has been proposed to play a role in oxygen sensing, and positively modulates the oxygen sensitivity of TASK-1 in oxygen sensing cells when those are excited by hypoxia (Lee et al. 2006b). Insulin stimulates Nox4 expression in adipocytes (Schroder et al. 2009) and IGF-1 has been found to induce Nox4 expression in VSMCs (Meng et al. 2008). Insulin induced expression of Nox4 leads to the oxidative inactivation of PTP1B, increasing the activity of other kinases (Mahadev et al. 2004). Furthermore, Nox4-mediated oxidation and inactivation of PTP1B in the ER serves as a regulatory switch for EGF receptor trafficking and specifically acts to terminate EGF signaling (Chen et al. 2008). Several reports have shown that Nox4-derived ROS are responsible of mediating proliferation and survival in response to different stimuli. Treatment of VSMCs with urokinase plasminogen activator results in Nox4-mediated growth and survival (Menshikov et al. 2006); Nox4 is also involved in proliferation in hypoxia-mediated activation of pulmonary adventitial fibroblasts (Li et al. 2008). Growth and survival effects of Nox4 activation have been reported to be mediated by AKT in mesangial cells stimulated with Ang II (Angiotensin II) (Gorin et al. 2003), and also through the oxidation–mediated activation of SRC (Block et al. 2008). In pancreatic cells Nox4 also has an anti-apoptotic role, since the depletion of Nox4 or ROS promotes apoptosis (Vaquero et al. 2004); moreover, in pancreatic cancer, Nox4 promotes activation of JAK/STAT pathway in order to enhance the growth response (Lee et al. 2007a). Nox4-associated ROS have also been implicated in progression through the G2/M checkpoint of the cell cycle via regulation of cdc25 phosphorylation (Yamaura et al. 2009). Moreover, Nox4 appears to have a role in promoting ERK activation in lung epithelial cells (Amara et al. 2007) in response to diesel exhaust particles (DEPs), a main component of particulate air pollution; Nox4 also plays a role in VEGF-stimulated ERK phosphorylation (Datla et al. 2007), and may also be involved in mediating ERK activation in response to PDGF (Wagner et al. 2007) and to arachidonic acid (Gorin et al. 2004). In the endothelium (Petry et al. 2006), Nox4 promotes proliferation, while in VSMCs Nox4 participates in the maintenance of their differentiated phenotype co-localized with α-SMA-based stress fibers in differentiated VSMC (Clempus et al. 2007). Additionally, Nox4 is involved in the differentiation of mouse embryonic stem cells (Li et al. 2006a) or fibroblasts into myocytes (Cucoranu et al. 2005).

In addition, in cardiac fibroblasts (Ellmark et al. 2005), lung (Sturrock et al. 2007) and pulmonary artery smooth muscle cells (Sturrock et al. 2006) and hepatocytes (Carmona-Cuenca et al. 2006) TGF-β induces increased expression of Nox4. Nox4 expression in response to TGF-β plays different roles depending on cell type. Treatment of pulmonary artery smooth muscle cells with TGF-β promotes proliferation through the expression of Nox4 in a Smad2/3-dependent manner (Sturrock et al. 2006). Nox4 has also been shown to mediate TGF-β-induced phosphorylation of retinoblastoma protein (pRb) and the eukaryotic translation initiation factor 4E (elF4E) binding protein-1, which regulate cell cycle progression and hypertrophy, respectively, in airway smooth muscle cells (Sturrock et al. 2007). TGF-β promotes fibroblast irreversible differentiation into myofibroblast (Cucoranu et al. 2005). In contrast, TGF-β-induced Nox4 expression is required for TGF-β-induced cell death in rat fetal hepatocytes (Carmona-Cuenca et al. 2008).

4.3. NOX4-derived ROS and their involvement in different diseases

A part from the different roles stated before, Nox family members have also been described to play a role in different chronic diseases that are often associated with tissue damage, fibrosis and in some cases probable genetic damage (Lambeth 2007), mainly due to the misregulation or absence of certain Nox isoforms. Nox4 had been proposed to contribute to tissue destruction in asthma (Hoidal et al. 2003). Interestingly, Nox4 induction in response to Urotensin II markedly increases ROS levels that activate ERK1/2, p38, JNK and AKT contributing to smooth muscle hypertrophy and proliferation associated to pulmonary hypertension (Djordjevic et al. 2005). Later on, it was described that TGF-β-induced expression of Nox4 in pulmonary artery smooth muscle cells, was probably involved in the development of pulmonary hypertension (Sturrock et al. 2006). Both Nox1 and Nox4 have also been implicated in mitogenesis and hypertrophy in the cardiovascular system, and therefore might participate in atherosclerosis and hypertension (Brandes 2003; Brandes and Schroder 2008; Frey et al. 2008). Nox4 also promotes angiogenesis and reduces endothelial apoptosis (Datla et al. 2007). Others studies have implicated Nox4 as a major source of reactive oxygen species (ROS) in many cell types and in kidney tissue of diabetic animals (Etoh et al. 2003; Block et al. 2009).

There is a strong correlation between Nox expression, reactive oxygen species and mitogenic growth/cancer, cancer and rapidly proliferating cells frequently overproduce reactive oxygen (Lambeth 2007). In many of cases, the sources of this ROS are Nox enzymes. Nox4 has been shown to be expressed in melanomas (Yamaura et al. 2009), and recently it has been proposed that the induction of Nox4 expression mediated by AKT may be in part responsible for transformation of radial growth to vertical growth (i.e., noninvasive to invasive) melanoma (Govindarajan et al. 2007). Nox4 have been found to be expressed in pancreatic cancer cells (Vaquero et al. 2004; Lee et al. 2007a), glioblastoma (Shono et al. 2008), prostatic cancers (Tam et al. 2007), its expression was found to be up-regulated in thyroid cancer (Weyemi et al. 2009). Moreover, several lines of evidence suggest that Nox enzymes are involved in promoting fibrotic responses in different organs such as kidney, liver and heart, leading to adverse pathological events (Lambeth 2007). In cardiac fibrosis, not only Nox2 is expressed in human cardiac fibroblasts, they also express Nox1, Nox4 and Nox5, and Nox4 is also found in the cardiomyocytes (Lambeth 2007). Nox4 has been shown crucial for transdifferentiation of cardiac fibroblasts into myofibroblasts in response to TGF-β, and such fibroblast transdifferentiation is one of the hallmarks of cardiac fibrosis; moreover, inhibiting the Nox4 expression with specific siRNA suppressed ROS generation, fibroblast transdifferentiation and collagen production (Cucoranu et al. 2005). Moreover, Nox4 expression is up-regulated in cardiac tissue of dystrophic mice (Spurney et al. 2008). It has been observed that blockage of Nox4 prevents the stimulatory effect of Ang II on fibronectin accumulation and cell hypertrophy in renal fibrosis (Block et al. 2008). Activated pancreatic stellate cells (PSCs) play an important role in pancreatic fibrosis and inflammation, where oxidative stress is implicated in the pathogenesis, these cells have high levels of expression of Nox1, Nox2 and Nox4 (Masamune et al. 2008). Recently, it has been described that in pulmonary fibrosis TGF-β induces Nox4 expression and its ROS production is required for myofibroblast differentiation, synthesis of ECM proteins and contractility during the progression of lung fibrosis (Hecker et al. 2009). Chronic liver diseases are characterized by increased ROS production as well as decreased activity of antioxidant systems, resulting in oxidative stress, which is commonly detected in patients with alcohol abuse, hepatitis C virus infection (HCV), iron overload and chronic cholestasis, as well as in most types of experimental liver fibrogenesis. In these conditions, oxidative stress is not only a consequence of chronic liver injury but also significantly contributes to excessive tissue remodelling and fibrogenesis. In the liver, activated hepatic stellate cells are the major source of extracellular matrix, and they are the main fibrogenic cells involved in

liver fibrosis (De Minicis and Brenner 2007). It has been shown that HCV induces Nox4 expression, which can contribute to ROS production and may be related to HCV-induced liver disease (Boudreau et al. 2009). Moreover, following ischemia/reperfussion (I/R) injury to the liver, results in alterations in Activating Protein 1 (AP1) DNA binding activity and activation of Nox2 and Nox4, which promote enhanced proliferation and caspase-mediated damage; whether enhanced NADPH oxidase-dependent ROS production by the liver is a consequence of enhanced AP-1-mediated damage, or instead acts as an effector of AP1, remains unclear (Marden et al. 2008).

5. EMT

5.1. EMT definition

In a recent review the epithelial and mesenchymal tissues are defined as follows: "**epithelial tissues** line the cavities of the human body and are the main tissue constituent of many glands. Epithelial tissues perform a variety of functions, including protection, secretion, absorption, filtration, and diffusion. They are composed of continuous sheets of polarized cells connected by strong cell-to-cell and cell-to-substratum adhesions. These cell-to-cell adhesive structures, which include adherens junctions, tight junctions, gap junctions, and desmosomes, ensure tight lateral connections between adjacent epithelial cells. Epithelial cells are also anchored basally to basal lamina (or basement membrane), which in concert with the lateral adhesive junctions maintains epithelial apical–basolateral polarity, localized distribution of cadherin and integrin adhesion molecules, and polarized organization of the actin cytoskeleton. **Mesenchymal tissues** are loosely organized and are composed of mesenchymal cells, which are typically not in contact with a basal membrane, exhibit disorganized adhesive structures, form only weak contacts with neighboring cells, and exhibit spindle-like morphology with front-to-back polarity. Unlike epithelial cells, which predominantly have a cytokeratin-rich network of intermediate filaments, mesenchymal cells have a vimentin-based network consisting of stress fibers and focal adhesions. Mesenchymal cells are highly capable of independent motility and secrete extracellular matrix degrading enzymes, and these properties likely facilitate their invasion through the basement membrane and surrounding tissues" (Hollier et al. 2009).

Epithelial- mesenchymal transition (EMT) is a normal embryonic process that occurs during various stages of embryogenesis as well as during numerous pathologic conditions, such as tissue fibrosis and cancer (Hollier et al. 2009). EMT can also be reversed via a process called mesenchymal–epithelial transition (MET), occurring after migration and homing into new sites within an embryo, during tumor progression or healing of fibrotic tissue (Heldin et al. 2009). EMT and its converse, MET, are concepts first defined by Elizabeth Hay, who later also described this cellular behavior during migration and the importance of the transient nature of this process (Hay 1990; Hay 1995). A number of distinct molecular processes are engaged in order to initiate EMT and enable it to reach completion. As recently reviewed, these mechanisms include activation of transcription factors, expression of specific cell-surface proteins, disruption of the intercellular adhesion complexes, reorganization and expression of cytoskeletal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs (Kalluri and Weinberg 2009). The different aspects of EMT generally occur sequentially, with inhibition of cell-cell contact occurring before cytoskeletal rearrangement and acquisition of a motile/invasive phenotype (Thiery 2003). Upon undergoing EMT, the cells acquire migratory and invasive properties that allow them to migrate through the extracellular matrix (Acloque et al. 2009). It is important to emphasize that EMT/MET refer to changes in cell shape, polarity and adhesive properties (Choi and Diehl 2009). Finally, it is worth to mention that cancer cells may pass through EMTs to differing extents, with some cells retaining many epithelial traits while acquiring some mesenchymal ones and other cells shedding all vestiges of their epithelial origin and becoming fully mesenchymal (Kalluri and Weinberg 2009). As mentioned, the EMT program is often considered a transient and reversible process; in this line of evidence, studies of embryonic development have demonstrated that the mesenchymal cells of the mesoderm can give rise to epithelial organs, for instance the kidney by undergoing a MET process (Davies 1996).

Several studies now provide direct evidence that an EMT process occurs in mouse and human carcinomas (Brabletz et al. 2001; Trimboli et al. 2008). In cancer, the epithelial tumor cells become more invasive after undergoing EMT and access the circulatory system through intravasation, resulting in dissemination of cancer cells to distal loci from the primary tumor. Consequent metastatic colonization of secondary sites by cancer cells is thought to involve the reverse MET process (Hugo et al. 2007). Some authors argue that the MET events certainly take place during metastasis formation as suggested by cell morphology and the re-expression of E-cadherin expression (Bukholm et al. 2000). This reversibility in gene expression suggests that transcriptional regulation rather than irreversible genetic loss may confer a selective advantage for breast cancer cell progression (Acloque et al. 2009). However, there is also evidence that tumor invasion can occur in the absence of EMT. Some studies show that expression of podoplanin promotes an alternative pathway of tumor cell invasion in the absence of EMT (Wicki et al. 2006). There are several lines of evidence suggesting that many invasive and metastatic carcinomas have not undergone a complete transition to a mesenchymal phenotype or even lack signs of EMT, and that invasive carcinomas do not invade adjacent connective tissue as individual mesenchymal-like cells. These carcinoma cells invade as multicellular aggregates or clusters (Friedl and Wolf 2003). In fact, it has been described that hepatocytes can migrate as cohorts in response to TGF-β (Biname et al. 2008). Hu's group propose that cancer cells with an EMT phenotype can invade into adjacent connective tissues, and intravasate; but they are unable to form metastatic nodules in the lung even when they are directly injected into the blood circulation. Cancer cells without an EMT phenotype cannot invade into adjacent connective tissue, but they have the ability to form metastatic nodules in the lung when they are intravenously injected. More importantly, cancer cells with a mixed EMT and non-EMT phenotype can complete the entire process of spontaneous metastasis (Tsuji et al. 2009).

5.2. EMT characteristics

EMT can be triggered in a context-specific manner by various extracellular stimuli. Many secreted molecules, such as Hedgehog, EGF, HGF, and members of the TGF-β, Wnt, FGF, and insulin-like growth factor families, which all act through the RAS/MAPK or PI3K/AKT pathway, result in the induction of Snail expression (Barrallo-Gimeno and Nieto 2005), therefore inducing EMT. For example, members of the TGF-β family are potent inducers of EMT in many cell types (Xu et al. 2009). Furthermore, the TGF-β/Smad pathway also cooperates with RAS, Notch and Wnt signaling in inducing Snail expression in development and in tumor metastasis (Barrallo-Gimeno and Nieto 2005).

Change in expression of cadherins is the prototypical epithelial cell marker of EMT, the so-called cadherin switches. E-cadherin is expressed in epithelial cells, and its expression is decreased during EMT in embryonic development, tissue fibrosis, and cancer (Hay and Zuk 1995). Interestingly, loss of E-cadherin function promotes EMT (Kalluri and Neilson 2003). The cadherin switch from E-cadherin to N-cadherin, which is expressed in mesenchymal cells, fibroblasts, cancer cells, and neural tissue, has often been used to monitor the progress of EMT during embryonic development and cancer progression (Zeisberg and Neilson 2009). Typical mesenchymal markers expressed in cells that have undergone EMT are: 1) Fibroblast-specific protein 1(FSP1, also known as S100A4 and MTS-1) is a member of the family of Ca^{2+}-binding S100 proteins (Strutz et al. 1995), which is detected in cells that have suffered EMT in cancer and fibrogenesis (Iwano et al. 2002); in the same line of evidence FSP1 itself facilitates EMT in adult epithelial cells and cancer cells (Xue et al. 2003). 2) Vimentin is commonly used to identify cells undergoing EMT in cancers (Yang et al. 2004). 3) α-SMA (Smooth Muscle Actin) is also expressed in mesenchymal cells after an EMT process (Zeisberg et al. 2007b).

Other mesenchymal markers that are up-regulated during an EMT process are fibronectin, matrix metalloproteinases and N-cadherin (Thiery 2003). Additionally, of the principal basement membrane constituents type IV collagens, laminin, and sulfated proteoglycans that are downregulated during EMT, laminin is best established as a biomarker of the process (Colognato and Yurchenco 2000); by contrast, upregulation of laminin 5 (α3β3γ2) is associated with EMT in cancer and in tissue fibrosis, and its expression is linked to EMT in breast carcinomas of the ductal type**,** hepatocellular carcinoma**,** and oral squamous carcinoma as recently reviewed in Zeisberg and Neilson (2009).

A number of transcription factors are capable of driving EMT, including several zinc finger homologs (e.g., Snail1, Snail2) (Nieto 2002), basic helix-loop-helix transcription factors (e.g., Twist and E47) (Ansieau et al. 2008), and ZEB family members (ZEB1, ZEB2/SIP1) (Eger et al. 2005). These factors suppress E-cadherin expression and promote EMT during cancer progression (Peinado et al. 2007)**.** Furthermore, a number of these EMT-inducing transcription factors are up-regulated during the progression of human malignancies, including breast cancer (Hollier et al. 2009). Overexpression of Snail, Twist, or Slug, the major regulators of EMT has been observed in primary HCCs, and co-expression of Snail and Twist correlate with the worst prognosis of HCC; in the same study they show that overexpression of Snail or/and Twist in Huh-7 cell line (a non-invasive cell line) induced EMT and invasiveness/metastasis, whereas knockdown of Twist or Snail in Mahlavu (an invasive cell line) reversed EMT and inhibited invasiveness/metastasis (Yang et al. 2009). As recently reviewed by Thiery et al. (2009), the classically known EMT inducers can be divided in two groups depending on their effects on the E-cadherin promoter. Snail, ZEB, E47, and KLF8 factors bind to and repress the activity of the E-cadherin promoter, whereas factors such as Twist, Goosecoid, E2.2, and FoxC2 repress E-cadherin transcription indirectly.

For example, Snail directly represses the expression of E-cadherin (Batlle et al. 2000; Bolos et al. 2003), which, in turn, leads to the disruption of adherens junctions. Additionally, Snail proteins repress a spectrum of genes involved in maintaining epithelial structure and function, including genes encoding claudins and occludin, major transmembrane components of tight junctions, as well as genes important for apicobasal polarity such as Crumbs3 and Discs large (Moreno-Bueno et al. 2008) . Snail expression also results in decreased expression of a subset of cytokeratins, i.e. cytokeratin 17, 18, 19 and 20, thus affecting the epithelial cytoskeletal organization (Ikenouchi et al. 2003; De Craene et al. 2005), in hepatocytes Snail represses HNF4α, a key factors of hepatic differentiation (Cicchini et al. 2006). As it has recently been reviewed, while repressing epithelial gene expression, Snail proteins activate the expression of the mesenchymal proteins fibronectin, vitronectin and N-cadherin, the extracellular matrix proteins collagen type III and V, and proteins involved in migration and invasion, such as RhoB, plasminogen activator inhibitor-1 and matrix metalloproteinases (Xu et al. 2009). Finally, Snail also promotes the activation of metalloproteases, MMP2, MMP3, and MMP9, which help to degrade the basal membrane (Jorda et al. 2005; Miyoshi et al. 2005).

Twist proteins are essential for proper gastrulation, mesoderm formation, and neural crest migration during development, which represent a typical EMT event (Yang et al. 2004). Ectopic expression of Twist2 induces EMT of Madin-Darby canine kidney cells (MDCK) cells by repressing expression of E-cadherin, α-catenin, occludin, and claudin-7, and promoting expression of vimentin and N-cadherin (Ansieau et al. 2008). ZEB2 directly represses the expression of the tight junction proteins claudin-4 and ZO-3, ZEB2 also suppresses the expression of the desmosome protein plakophilin-2 (Vandewalle et al. 2005); while it induces the expression of the mesenchymal proteins vimentin (Bindels et al. 2006), N-cadherin (Vandewalle et al. 2005) and matrix metalloproteinase-2 (Taki et al. 2006); consequently, ZEB proteins promote cell migration and induce invasion.

A key component of epithelial junctions is β-catenin, which is part of the protein complex that connects cadherins to the actin cytoskeleton at adherens junctions. In response to Wnt, β-catenin is translocated from the cell membrane to the nucleus, where it can regulate gene expression and induce EMT (Nelson and Nusse 2004). Interestingly, Snail interacts with β-catenin and stimulates its transcriptional activity (Stemmer et al. 2008). Disruption of tight junctions can also be mediated through EphrinB1, when it associates to Par6, it prevents Par6 binding to Cdc42 (Lee et al. 2008). Furthermore, Notch signaling has also been shown to contribute to EMT in both tumor progression and cardiac development (Wang et al. 2009b).

In addition, integrin signaling facilitates EMT (Li et al. 2003), and various integrins are expressed on both epithelial and mesenchymal cells. As a result, integrins in general have limited utility as generalized biomarkers for EMT. In colon carcinoma, only cancer cells that have undergone EMT to a metastatic phenotype express high levels of β6 integrin; kidney fibrosis is associated with increased α5 integrin expression (White et al. 2007). Moreover, increased expression of α5 integrin also correlates with the metastatic potential of melanoma cells and EMT (Qian et al. 2005), suggesting that α5 integrin plays a role in EMT.

Finally, microRNAs have also been recently found to be involved in the induction of EMT: the miR-200 family is markedly downregulated in TGF-β1-induced EMT and in cancer cell lines that displayed an EMT phenotype (Gregory et al. 2008); conversely, TGF-β1-induced EMT in keratinocytes is associated with induction of miR-21 (Zavadil et al. 2007). Twist-induced EMT is associated with induction of miR-10b in breast cancer cells (Ma et al. 2007a).

5.3. EMT generates stem cell like properties and promotes survival

Induction of EMT in HMLE or MCF10A cells either by over-expression of Twist, Snail or RAS, or by treatment with TGF-β is coincident with the apparition of a CD44+/CD24- population, which expression is associated with both human breast CSCs and normal mammary epithelial stem cells. Moreover, cells generated by an EMT process acquired the ability to form mammospheres, another attribute of mammary stem cells (Mani et al. 2008). It has been recently shown that a metastatic breast cancer cell line to the lung requires Wnt signaling for maintenance of the dedifferentiated epithelial phenotype consistent with EMT and cancer cell self-renewal. Wnt signaling maintains the expression of CD44high/CD24low, and the ability of these cells to metastasize through the expression of Slug and Twist (DiMeo et al. 2009). In agreement with a link between EMT and stemness, over-expression of Ladybird homeobox 1 (LBX1), a developmentally regulated homeobox gene, directs expression of the known EMT inducers ZEB1, ZEB2, Snail1, and TGF-β2 in mammary epithelial cells; these events promote expression of mesenchymal markers, enhance cell migration, and increase CD44high/CD24low progenitor cell population (Yu et al. 2009). Furthermore, the sine oculisrelated homeobox 1 homolog (Six1) homeoprotein, expressed during early embryogenesis but lost in most adult tissues is essential for the development of numerous organs, recent evidence demonstrates that Six1 plays a role in cellular migration and invasion during embryogenesis (Xu et al. 2003a; Zheng et al. 2003) through a mechanism that may involve an EMT. Recently, it has been described that Six-1 induces EMT and promotes stem/progenitor cell phenotype in the mouse mammary gland and in Six1-driven mammary tumors (McCoy et al. 2009). Moreover, Six1-induced experimental metastasis is dependent on its ability to activate TGF-β signaling (Micalizzi et al. 2009).

In addition to regulating the expression of epithelial or mesenchymal genes, Snail also regulates genes required for cell survival, which are frequently intertwined with EMT during embryonic development or in pathological conditions (Barrallo-Gimeno and Nieto 2005), this is the case in fetal hepatocytes (Gotzmann et al. 2002; Valdes et al. 2002) and the neural crest (Vega et al. 2004). Snail promotes the inhibition of proliferation through suppression of cyclin D proteins (Vega et al. 2004) and the protection from cell death (through suppression of expression of caspases, DNA fragmentation factor, and Bcl-interacting death agonist) induced by withdrawal of serum, pro-apoptotic signals or DNA damage (Kajita et al. 2004; Martinez-Alvarez et al. 2004; Vega et al. 2004). Other examples are that TGF-β-induced EMT in mammary epithelial cells also confers resistance to ultraviolet-light-induced apoptosis (Robson et al. 2006). Similarly, Slug inhibits apoptosis (Wu et al. 2005) and Twist can also inhibit apoptosis during development and has pro-survival functions in neuroblastoma (Puisieux et al. 2006).

Additionally, overexpression of Snail or Slug in ovarian cancer cells promotes chemo-resistance and acquisition of stem cell-like phenotype (Kurrey et al. 2009). Different works have shown that EMT promotes acquisition of chemoresistance in breast cancer and lung carcinoma cell lines, reviewed in Hollier et al.(2009): for example, suppression of Twist in the lung carcinoma cell line A549 resulted in chemosensitivity to cisplatin; EMT induced by EGFR signaling has been linked to tamoxifen resistance and increased invasiveness of MCF-7 cells; it has been shown that chemoresistance correlates with higher levels of Twist and AKT2 in breast cancer lines resistant to paclitaxel; similarly, overexpression of Snail or Slug in MCF-7 cells protects them from apoptosis induced by the DNA-damaging chemotherapeutic agent doxorubicin. Moreover, it has been shown that Snail and Slug promote resistance to radiation and paclitaxel by effectively inactivating p53-mediated apoptosis (Kajita et al. 2004; Kurrey et al. 2009). In addition, studies of non-small-cell lung carcinoma have shown that an EMT-like phenotype has been associated with sensitivity to the EGFR inhibitor erlotinib (Thomson et al. 2005); similar results have been observed in HCC cell lines (Fuchs et al. 2008). However it has recently been shown that Salinomycin selectively kills breast CSCs. Although the mechanism of action for salinomycin is not yet clear, it appears that it might induce terminal epithelial differentiation accompanied by cell-cycle arrest rather than trigger cytotoxicity (Gupta et al. 2009).

Therefore, cells that undergo EMT gained three critical capabilities: invasiveness, resistance to p53-mediated apoptosis, and a self-renewal program, that together define the functionality and survival of metastatic cancer stem cells. Additionally, EMT confers immunosuppression and there is a tight association between EMT and cancer-associated inflammation (Thiery et al. 2009)

5.4. EMT Types

A proposal to classify EMTs into three subtypes based on the biological and biomarker context in which they occur was discussed at a 2007 meeting on EMT in Poland and at a subsequent conference in March 2008 at Cold Spring Harbor Laboratories. Vastly, Type 1 EMTs occur during implantation, embryogenesis, and organ development; the type 2 EMT begins as a repair-associated event in adult tissues; the type 3 EMTs occur as a result of genetic and epigenetic changes in cancer cells and promote invasion and spread of tumor cells, as well as subsequent emergence of metastatic tumor foci at sites distant from the primary tumor, this classification has been recently reviewed (Kalluri 2009; Kalluri and Weinberg 2009).

Type 1 EMT is associated with implantation, embryo formation, and organ development to generate diverse cell types that share mesenchymal phenotypes and biomarkers; it generates cells with a mesenchymal phenotype to create new tissue(s) with diverse functions. During development, EMT plays a critical role in generating the first set of mesenchymal cells, which are known as the primary mesenchyme. Subsequently, as tissue expands and specifications emerge, primary mesenchyme gives rise to secondary epithelia via MET (Kalluri 2009). The EMT associated with gastrulation is dependent on and orchestrated by canonical Wnt signaling, TGF-β superfamily proteins, notably Nodal and Vg1, and their deficiencies can lead to mesodermal defects due to the absence of functional EMTs; Wnts also cooperate with FGF receptors to help regulate an EMT associated with gastrulation. In the absence of the EMT process, gastrulation cannot occur, and development of the embryo does not progress past the blastula stage; as recently reviewed (Hollier et al. 2009; Kalluri and Weinberg 2009).

Type 2 EMT is associated with wound healing, tissue regeneration, and organ fibrosis; EMTs in this context begin as part of a repair-associated event to generate fibroblasts to reconstruct and repair tissue following trauma and/or inflammatory injury. This process ceases once repair is achieved and inflammation is attenuated. However, in the case of organ fibrosis, type 2 EMT can continue to respond to ongoing inflammation, leading eventually to organ destruction (Kalluri 2009). Organ fibrosis, which occurs in a number of epithelial tissues, is mediated by inflammatory cells and fibroblasts that release a variety of inflammatory signals as well as components of a complex ECM that includes collagens, laminins, elastin, and tenacins; such EMTs are found to be associated with fibrosis occurring in kidney, intestine (Iwano et al. 2002), liver (Zeisberg et al. 2007c), and lung (Willis and Borok 2007). FSP1, α-SMA, and collagen I have provided reliable markers to characterize the mesenchymal products (Zeisberg and Neilson 2009); both epithelial (Iwano et al. 2002) and endothelial cells (Zeisberg et al. 2007a; Zeisberg et al. 2008) are likely to be substrates for new fibroblasts in adult tissue, the fibroblasts formed should also show a stable phenotype on removal of the induction stimulus and with passage or time (Zeisberg and Neilson 2009). As recently reviewed, cells that have undergone a type 2 EMT process express the mesenchymal marker FSP1 and α-SMA, but concomitantly continue to have epithelial-specific morphology and molecular markers, such as cytokeratins and E-cadherin. Such cells are likely to represent intermediate stages of EMT, the behavior of these cells provided one of the first indications that epithelial cells under inflammatory stresses can advance to various extents through an EMT, creating the notion of "partial EMTs" (Kalluri and Weinberg 2009). TGF-β has proved to be an inducer of EMT, promoting type 2 EMT (Sato et al. 2003; Saika et al. 2004; Willis and Borok 2007; Meindl-Beinker and Dooley 2008); interestingly, BMP-7 functions as an endogenous inhibitor of TGF-β–induced EMT; systemic administration of recombinant BMP-7 to mice with severe fibrosis resulted in reversal of EMT and repair of damaged epithelial structures, with repopulation of healthy epithelial cells and restoration of organ function (Zeisberg et al. 2005). Different cell types in the liver have been shown to undergo an EMT process: cholangiocytes from rats with biliary fibrosis co-expressed epithelial and mesenchymal markers (Diaz et al. 2008; Omenetti et al. 2008); and hepatocytes both *in vitro* (Valdes et al. 2002) and *in vivo* induced fibrosis (Meindl-Beinker and Dooley 2008). Moreover, three different fate-mapping studies have provide data showing that hepatocytes (Zeisberg et al. 2007c), hepatic stellate cells (Yang et al. 2008b) or oval cells (Sackett et al. 2009) might be contributing to the fibrotic process in certain types of adult liver injury through an EMT/MET mechanism.

Type 3 EMT occurs in carcinoma cells that have already suffered genetic and epigenetic alterations, which make them more sensitive to EMT-inducing signals originated from the tumor-associated stroma (Kalluri and Weinberg 2009). In these cells the EMT process is not a mechanism for forming

fibroblasts, but a mechanism to transition and to form tumor epithelial cells for movement, invasion, and metastasis (Zeisberg and Neilson 2009); these cells invade and metastasize via the circulation, and once they find themselves in distant tissue, they form secondary tumors exhibiting an epithelial phenotype, through a MET process (Kalluri 2009). The idea that metastatic cancer cells have undergone an EMT process is based on the acquisition of mesenchymal markers such as Vimentin, FSP1 by epithelial carcinoma cells that have acquired metastatic properties (Thompson et al. 1994), nuclear localization of β-catenin and loss of epithelial cell adhesion molecules such as E-cadherin (Xu et al. 2003b). In HCC, Snail expression is induced and accelerates invasion activity by upregulating Metalloprotease (MMP) expression, resulting in portal invasion, intrahepatic metastasis, and poor prognosis (Miyoshi et al. 2005); induction of EMT has been observed in cultured hepatoma cell lines and it correlates with increased migration capacity (Bertran et al. 2009).

6. TGF-β

6.1. TGF-β signaling pathway

The transforming growth factor-β (TGF-β) family of cytokines has 33 members in humans, these include bone morphogenic proteins (BMPs), activin/inhibin, growth and differentiation factors (GDF)s, nodal, and anti-Müllerian hormone. TGF-β family members regulate numerous cellular processes that include cell proliferation, cell differentiation, apoptosis, extracellular matrix production, cell fate and migration. These cytokines play very important roles during development, as well as in normal physiological and disease processes. Disruption of TGF-β signaling has been implicated in the progression of diseases such as cancer, fibrosis and autoimmune disease (Pardali and Moustakas 2007; Ross and Hill 2008).

TGF-β is secreted as an inactive latent disulfide-linked homodimeric polypeptide that is bound to other extracellular proteins such as latent TGF-β binding proteins (LTBPs) that anchors the ligand in the extracellular matrix. The mature, bioactive ligand that consists of the processed C-terminal homodimeric polypeptide is produced upon proteolytic cleavage of the latent complex. The mature ligand binds directly to the protein core of a transmembrane proteoglycan receptor, β-glycan or TGF-β type III receptor. β-glycan function is not absolutely necessary for TGF-β signaling, since it is not expressed in all cell types and it can also be replaced by the related transmembrane glycoprotein named endoglin that acts as an accessory protein for the receptor complex (Pardali and Moustakas 2007). These two co-receptors form complexes with TGF-β receptors and mediate ligand presentation to them. Two related receptors transmit biological signals, the TGF-β type II receptor (TβRII) and TGF-β type I receptor (TβRI), also named activin receptor-like kinase 5 (ALK5). TβRII and TβRI form hetero-tetrameric complexes of two identical TβRII/TβRI receptor heterodimers, and bind to dimeric TGF-β. Upon oligomerization, the type II receptor phosphorylates and activates the type I receptor, which phosphorylates downstream effectors of the Smad family. The Smad family consists of eight members, which form three subfamilies: receptor-activated (R-)Smads, a single common-mediator (Co-)Smad (Smad4), and two inhibitory (I-)Smads (Smad6 and Smad7). There are five vertebrate R-Smads, Smad1, Smad2, Smad3, Smad5 and Smad8 and they are phosphorylated in response to different types of TGF-β family members. TGF- β, Nodal or Activin ligands lead to phosphorylation of Smad2 and Smad3, whereas the BMP and GDF ligands induce phosphorylation of Smad1, Smad5 and Smad8. However, in endothelial cells TGF-β ligand can phosphorylate Smad1, 5 and 8 through a receptor complex that contains the tissue-specific ALK1 type I receptor (Ross and Hill 2008; Heldin et al. 2009). TGF-β also induces non-Smad pathways, including mitogen-activated protein kinases (MAPK), phosphoinositide-3-kinase (PI3K), PP2A phosphatase (PP2A), and Rho GTPases (Rho). These non-smad signaling play different roles, they are involved in TGF-β-mediated biological responses but they can also regulate the canonical Smad pathway (Pardali and Moustakas 2007; Yu et al. 2008).

6.1.1. Smad dependent signaling

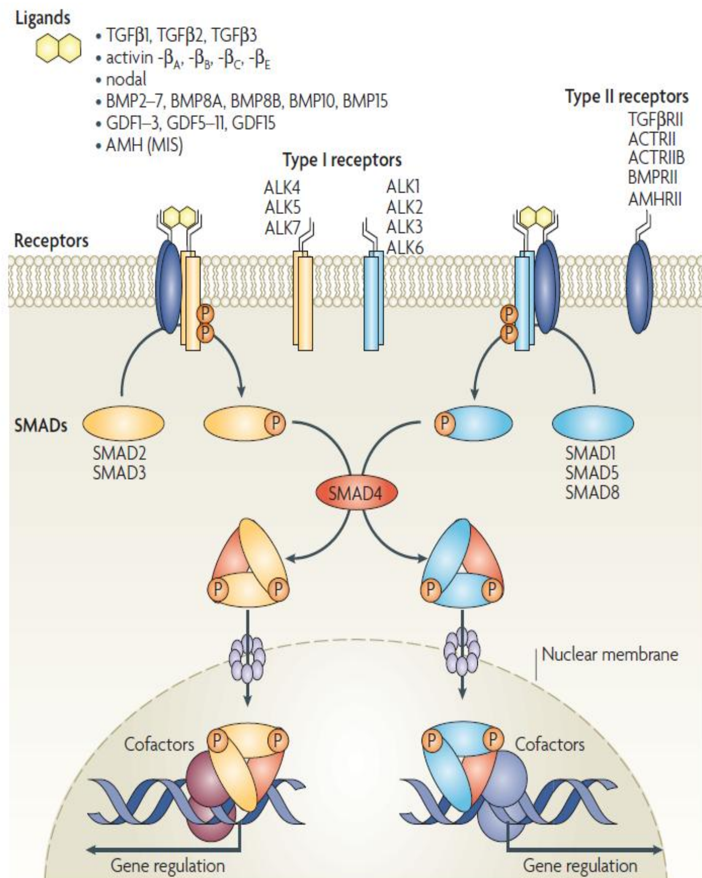


Figure 14. Canonical Smad signaling. From Schmierer and Hill, 2007.

The R-Smads and Smad4 have conserved amino and carboxyl regions termed the Mad homology (MH)1 and MH2 domain, respectively, which are separated by a more variable linker region. In the R-Smads, at the C-terminus there is a characteristic SSXS (Ser-Ser-X-Ser) motif that is phosphorylated by activated type I receptors. This motif is not present in Smad4. The I-Smads are structurally related to the R-Smads and Smad4 in their MH2 domain, but have a more divergent N-terminus. The MH1 domain of Smad4 and all the R-Smads, except the most common isoform of Smad2, has DNA-binding activity, but with low affinity. Smads also bind to GC-rich motifs. The MH1 domain of the R-Smads and Smad4 also contains a nuclear localization signal (NLS). The MH2 domain of the Smads is the effector region of the protein and is required together with the C-terminal portion of the linker domain, for transcriptional activity of the R-Smads and Smad4. The MH2 domain additionally mediates numerous protein–protein interactions including binding of the R-Smads and I-Smads to the type I receptor, formation of Smad–Smad complexes, Smad–SARA complexes and interactions of the Smads with numerous transcription factors, activators and repressors. The MH1 and MH2 domains are connected by the linker sequence, this region binds to ubiquitin ligases of the Smurf (Smad Ubiquitination Regulatory Factors) family, as well as it presents acceptor sites for regulatory phosphorylations by other signaling kinases as MAPK and cyclin-dependent kinases (CDKs) (Derynck and Miyazono 2008; Ross and Hill 2008).

TGF-β binding stabilizes the interaction of the TβRII dimer with two TβRI molecules, enabling TβRII to phosphorylate the TβRI on serine and threonine. The catalytically active TβRI phosphorylates the C-terminal serine residues of R-Smads, which as mentioned before in the case of TGF-β are Smad2 and Smad3. Once phosphorylated, R-Smads oligomerize with Smad4 forming trimeric protein complexes. These complexes are tranlsocated to the nucleus where they associate with other transcription factors in order to induce or repress gene expression. The Smads constitutively shuttle between the cytoplasm and nucleus, but their C-terminus phosphorylation leads to their accumulation in the nucleus. Within the nucleus, the Smad complex can dissociate and the phosphorylated R-Smads (phospho-R-Smads) are de-phosphorylated by nuclear phosphatases (such as PPM1A/PP2C), thus allowing the Smads to become available for export to the cytoplasm. This cycle continues for as long as active receptors are present (Pardali and Moustakas 2007; Clarke and Liu 2008). Smad activation is facilitated by the FYVE domain protein SARA which resides in endosomes and presents Smad2, and to a lesser extent also Smad3, to TGF-β receptors. Smads phosphorylation

decreases the affinity of the Smads for SARA. SARA is required for efficient Smad activation in response to TGF-β and directs the activated receptor toward clathrin-mediated endocytosis. Endofin, a structural homolog to SARA, binds TβRI and Smad4 and facilitates TGF-β signaling. Both endofin and SARA also have a negative role in signaling by binding the GADD34-PP1c phosphatase and recruiting it to the type I receptors, and thus deactivating them (Siegel et al. 2003a; Heldin et al. 2009). Other adaptors of Smad interaction with type I receptors are Axin and Dab2 among others. Axin is found in a complex with activated TβRI and Smad3, and facilitates Smad3 phosphorylation by TβRI, acting as a Smad stabilizer. Additionally, the PTB domain proteins Dab2 and Dok-1 both bind to TβRI, TβRII and the R-Smads (Kang et al. 2009).

The common mediator Smad4 is needed for most of the signaling effects of R-Smads. However, recent findings suggest that Smad4 may not be obligatory. It has been demonstrated that IKKα binding to Smad2 and Smad3 is essential for keratinocyte differentiation and growth arrest in response to TGF-β (Descargues et al. 2008). In the same argumentative line, it was recently identified that TIF1g, also known as Ectodermin, acts as a transcriptional partner of activated R-Smads in competition with Smad4 (Itoh and ten Dijke 2007).

As mentioned above, TGF-β stimulates the activation of SMAD proteins that accumulate in the nucleus and control transcription of a large number of target genes. The DNA-binding affinity of the Smads to the Smad Binding Element (SBE), GTCT or its reverse complement AGAC, is relatively weak with multimers of SBE sites being required for ligand-induced activation, and Smad2 lacks any DNA-binding activity due to an additional exon within its MH1 domain. It is generally thought that Smad/Smad4 complexes bind to DNA through Smad4. Therefore, in order to achieve high affinity and specific recruitment to DNA, the Smad proteins synergise with other DNA-binding factors (Siegel et al. 2003a; Ross and Hill 2008). The transcriptional responses to TGF-β depend on the cellular context, since every cell type contains a different variety of transcription factors which also vary depending on their differentiation state. Smads cooperate with a multitude of other transcription factors, for instance, FoxH1 and Mix family transcription factors. Foxo3, Smad3 and Smad4 interact in response to TGF-β and are recruited to the distal region of the p21 promoter. Smad3 also forms a transcriptional repressor complex with the basic leucine-zipper factor ATF3, a complex that appears to inhibit the expression of the ID genes in response to TGF-β. Other transcription factors reported to co-operate with the TGF-β-regulated R-Smads include members of the activating protein-1 (AP1) family of transcription factors, nuclear factor-kappaB (NF-κB), runt-related transcription factor-2 (Runx2), signal protein-1 (Sp1) and others, reviewed in Ross and Hill (2008).

The major mechanism by which Smads regulate transcription is through modulation of chromatin structure. Post-translational modification of the amino-terminal regions or ‘tails’ of histones by acetylation is associated with a permissive chromatin environment and transcriptional activation. Acetylation of lysine residues is predicted to neutralize the basic charge of the histones, thus weakening their contacts with DNA and changing interactions with both other nucleosomes and nucleosomal-binding proteins, enabling transcription factors and the transcription machinery to access DNA. Consistent with the transcriptional activating role of histone acetylation, many transcription co-activators have histone-acetyltransferase activity. CBP and p300 proteins play an important role in the regulation of TGF-β-induced transcription. In addition, Smads recruit co-repressors to both repress the basal level of transcription of target genes normally induced by TGF-β and to actively repress some TGF-β responsive genes. Histone deacetylases (HDACs) remove acetylation groups from histones tails allowing strong ionic interactions between the histones and DNA. This results in a more restrictive chromatin environment and prevents the transcription machinery from binding to the

promoter DNA. Thus, deacetylated histones are associated with repressed transcription. HDACs recruited directly by Smads or by associated co-repressors have been implicated in Smad-mediated repression (Ross and Hill 2008). Depending on the transcription partner, the SMAD complex will recruit co-activators such as p300, CBP or SMIF or co-repressors such as p107, which will determine whether the target gene is activated or repressed. Other co-repressors such as SKI, SNON, TGIF, EVI1 and SIP1 attenuate SMAD-mediated transactivation (Siegel et al. 2003a). Ski/SnoN interact directly with Smad2, Smad3 and Smad4. Ski confers resistance to the TGF-β cytostatic response and when bound to the nuclear Smad complex on target genes, recruits co-repressors of the N-CoR family and histone deacetylases, thus blocking the positive transcriptional activity of Smads. Moreover, the DNA-binding site recognized by Ski and SnoN is the same as the consensus Smad3/Smad4-binding element, and SnoN has been suggested to repress transcription from these elements by forming a complex with Smad4 (Itoh and ten Dijke 2007; Pardali and Moustakas 2007).

6.1.2. Non-Smad pathways

During the last few years, increasing evidence has been recollected in favor of TGF-β signaling through non-Smad pathways. These alternative pathways regulate the cellular response to TGF-β either alone or in conjunction with the canonical Smad pathway, or by regulating Smad activity. The TβRII and TβRI receptors are also phosphorylated on tyrosines, probably as a result of the abilities of their kinases to phosphorylate on tyrosine, in addition to serine and threonine. The TβRII cytoplasmic domain is autophosphorylated on three tyrosines, which might have auto-regulatory roles in defining the kinase activity of TβRII (Lawler et al. 1997). The TβRII can also be phosphorylated on tyrosine residues by SRC, enabling TβRII to recruit Grb2 (Growth Factor Receptor Bound 2) and Shc (Src-homology-2 domain) through their SH2 domains, and to contribute to TGF-β induced activation of the p38 mitogen-activated protein (MAP) kinase pathway. TGF-β also induces tyrosine phosphorylation of TβRI, although it is unclear whether this is mediated by the TβRI or TβRII kinases (Kang et al. 2009).

Rho-like GTPases in TGF-β signaling: The Rho-like GTPases, including RhoA, Rac and Cdc42, play important roles in controlling dynamic cytoskeletal organization, cell motility, and gene expression through a variety of effectors. TGF-β rapidly activates RhoA-dependent signaling pathways to induce stress fiber formation and mesenchymal characteristics in epithelial cells. TβRI localizes to tight junctions by interacting with the integral membrane protein occludin. Par6, a scaffold protein regulating epithelial cell polarity, interacts with TβRI at tight junctions. Upon TGF-β stimulation the assembly and accumulation of TβRI/TβRII complexes at tight junctions occurs, where TβRII phosphorylates Par6 at the serine residue 345. This phosphorylation allows Par6 to recruit Smurf1. The Par6/Smurf1 complex then mediates localized ubiquitination and turnover of RhoA at cellular protrusions, which enables TGF-β-dependent dissolution of tight junctions and rearrangement of the actin cytoskeleton. The Smurf1-mediated degradation of RhoA is a localized event and requires the presence of Smurf1 at lamellipodial- and filopodial-like protrusions (Ozdamar et al. 2005). Besides RhoA, TGF-β can also induce activation of the Cdc42 GTPase. Activation of Cdc42 by TGF-β appears to be independent of Smads. There is physical interaction between Cdc42 and cell surface TGF-β receptor complexes, together with a cluster of proteins involved in the Cdc42 and PAK network. Smad7 appears to be required for TGF-β-mediated Cdc42 activation (Zhang 2009). In addition, Smad3/Smad4 cooperate with Rho and p38 signaling to drive expression of NET1 and

tropomyosins, respectively, which are important for long-term establishment of actin stress fibers (Moustakas and Heldin 2005).

TGF-β-induced ERK activation and tyrosine phosphorylation: Tyrosine phosphorylation of TβRI is needed for activation of the ERK/MAP kinase pathway in response to TGF-β. The tyrosine phosphorylation sites (Y259, Y336, and Y424) permit the binding of the adaptor protein ShcA via its PTB domain to the receptor, which recognizes phosphotyrosine motifs. This interaction enables TβRI to phosphorylate Shc on tyrosine and serine, resulting in recruitment of Grb2 and SOS (Son of Sevenless) to Shc, leading to the activation of ERK (Lee et al. 2007b). TβRII can also be phosphorylated by SRC, a non-RTK, on Y284, which can also serve as a docking site for the recruitment of Grb2 and Shc, thereby bridging TβRII to MAPK activation (Galliher and Schiemann 2007). The kinetics of ERK phosphorylation induced by TGF-β varies with cell types and culture conditions. In some cell lines, a delayed response of ERK to TGF-β was reported, typically with the peak of ERK phosphorylation occurring hours after ligand stimulation, suggesting an indirect response requiring protein translation (Simeone et al. 2001). In contrast, in other types of cells, activation can occur rapidly within 5-10 min of TGF-β stimulation, which is comparable to the time course of ERK activation by mitogenic factors such as EGF (Olsson et al. 2001).

TGF-β-induced JNK/p38 activation: These MAPK pathways are activated by TGF-β independently of Smads, but require TGF-β-activated kinase 1 (TAK1). TRAF6 was known to play an important role in the activation of TAK1 in interleukin-1 receptor (IL-1R)- and Toll like receptors (TLRs)-mediated signaling pathways. It was only recently that it was found to be crucial for TGF-β-induced activation of the TAK1-JNK/p38 pathways. TRAF6 has a C-terminal TRAF domain and its N-terminal domain contains an E3 ligase RING finger domain and several Zn fingers. TRAF6 associates with TβRII and TβRI through its C-terminal TRAF domain. Binding of TRAF6 to activated TGF-β-receptor complex induces Lys63-linked polyubiquitination of TRAF6, and promotes the association between TRAF6 and TAK1, as well as TAK1 polyubiquitination which promotes its kinase activity. Activated TAK1 then phosphorylates and activates MKK3/6 or MKK4, which in turn activates p38 and JNK, respectively. Smad7 acts as a scaffolding protein to facilitate the activation of this MAP kinase cascade. Although the kinase activity of TβRI is not required for interaction with TRAF6, it is required for polyubiquitination of TRAF6 and activation of downstream kinase pathways induced by TGF-β. TRAF6/TAK1/JNK/p38 pathway is essential for both TGF-β-induced apoptosis and TGF-β-induced EMT (Sorrentino et al. 2008; Yamashita et al. 2008a).

PI3K/AKT pathway in TGF-β/Smad-mediated responses: TGF-β can activate PI3K, as indicated by the phosphorylation of its downstream effector AKT. This activation might be independent of Smad2/3 activation. It has been proposed that the TβRII receptor is constitutively associated with p85, the regulatory subunit of PI3K, while the association of the TβRI with p85 requires TGF-β stimulation. The association between TGF-β receptors and p85 is not direct, but the kinase activities of the TGF-β receptors are required for TGF-β-induced PI3K activation (Wilkes et al. 2005). In addition, TGF-β may also induce activation of PI3K indirectly through TGF-β-induced transactivation of the EGFR pathway (Vinals and Pouyssegur 2001; Murillo et al. 2005). The PI3K/AKT pathway is another non-Smad pathway contributing to TGF-β-induced EMT, which might be mediated, at least in part, by a downstream effector of AKT, mTOR, which is a key regulator of protein synthesis via phosphorylation of S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Although Rapamycin, an inhibitor of mTOR, does not affect TGF-β-induced morphological changes, it inhibits the enhanced migration and invasive behavior associated with TGF-β-induced EMT. Therefore, the TGF-β-induced translation pathway through PI3K/AKT/mTOR may complement the

transcription pathway mediated by Smads in TGF-β-induced EMT (Zhang 2009). However, in many other TGF-β-induced responses, the PI3K/AKT pathway antagonizes Smad-mediated effects. For example, activation of PI3K/AKT protects cells from TGF-β-induced apoptosis and growth inhibition (Valdes et al. 2004; Song et al. 2006). It has been proposed that the physical interaction between AKT and Smad3 prevents TβRI-mediated phosphorylation and nuclear localization of Smad3, thereby resulting in inhibition of Smad3-mediated transcription (Conery et al. 2004; Remy et al. 2004). In order to induce cell cycle arrest, TGF-β induces p15INK4B and p21CIP1 expression, for which a transactivation complex containing Smad3, Smad4, and FoxO family of transcription factors is required. It has been described that AKT can inhibit nuclear localization of FoxO proteins by phosphorylating them, and avoiding their involvement in transcription factor complexes (Seoane et al. 2004). Finally, it is worthy to note that TGF-β might play a dual role on PI3K/AKT regulation since it has been shown that TGF-β might down-regulate PI3K/AKT signaling activity through Smad-dependent expression of the lipid phosphatase SHIP, which may account for the transient nature of TGF-β-induced phosphorylation of AKT (Valderrama-Carvajal et al. 2002).

6.1.3. Regulation of TGF−β pathway

TGF-β signaling is modulated by several feedback mechanisms. The Smad shuttle in and out of the nucleus is regulated by cycles of phosphorylation and dephosphorylation of the R-Smads. Inhibitory (I)-Smads, Smad6 and 7, were originally found to compete with R-Smads for binding to activated type I receptors, thereby inhibiting R-Smad phosphorylation. But they also function through other mechanism such as interaction with Smad4, preventing R-Smad/Smad4 complex formation; they recruit phosphatases that dephosphorylate and inactive TGF-β receptors. In addition, they enhance type I receptor ubiquitylation and subsequent receptor degradation through recruitment of WW and HECT domain E3-ubiquitin ligases Smurf1 and 2; and direct repression of Smad-induced transcriptional responses (Itoh and ten Dijke 2007; Pardali and Moustakas 2007; Ross and Hill 2008). These post-translational modifications regulate the stability and availability of the receptors and the activation of both Smad and non-Smad signaling and, consequently, act as key determinants of the cellular response to TGF-β.

Ubiquitylation

Ubiquitylation and ubiquitin-mediated protein degradation define the stability and turnover of many proteins, including cell-surface receptors. Ubiquitylation occurs through sequential actions of E1, E2 and E3 ubiquitin ligases that provide the specificity and last step in the ubiquitylation process. A multitude of HECT domain E3 ligases and at least one RING domain E3 ligase can interact with and ubiquitinate Smads and TGF-β receptors, and have both negative and positive effects on the transcriptional responses of the TGF-β signaling pathways. Smad ubiquitination regulatory factors (Smurf) E3 ligases, Smurf1 and Smurf2, bind with different affinity to R-Smads. Smurf1 specifically targets Smad1/5, Smurf2 appears to have a broader Smad specificity. Interestingly, while Smurf2 binds to both Smad2 and 3, it does not degrade Smad3; instead, it induces the degradation of proteins such as SnoN that interact with the Smad3/Smurf2 complex (Itoh and ten Dijke 2007). Two other Smurf-related proteins, NEDD4-2 (neuronal precursor cells expressed, developmentally downregulated 4-2) and WWP1/TiuL1 (WW domain containing E3 ubiquitin ligase 1/TGIF-interacting ubiquitin ligase 1), have also been implicated in negatively regulating the TGF-β and/or BMP signaling pathways through degradation of R-Smads. ROC1 (regulator of cullins-1), a RING-

finger domain protein that forms part of the Skp/cullin/F-box (SCF) E3 ligase complex, can interact with Smad3 causing its ubiquitination and degradation (Ross and Hill 2008).

The inhibitory Smads, Smad6 and Smad7 interact with a variety of WW-HECT domain E3 ligases, including Smurf1, Smurf2, WWP1 and NEDD4-2. Smurf1 and Smurf2 redistribute the Smad7 from its predominantly nuclear localization in unstimulated cells into the cytoplasm and plasma membrane. The Smurfs also enhance Smad7 ubiquitination and can mediate a low level of Smad7 degradation. Smad7 functions as an adaptor and regulator of the Smurf/UbcH7 ubiquitin machinery to degrade the TGF-β receptor complex. I-Smads also interact with the E3 ligase Arkadia, Smad7/Arkadia complex stimulates poly-ubiquitination and degradation of Smad7, but does not affect the levels of TGF-β receptors (Ross and Hill 2008). The deubiquitylating enzyme UCH37 was found to associate with Smad7, reversing Smad7/Smurf2-mediated ubiquitylation and inhibiting the degradation of the TGF-β receptor (Kang et al. 2009).

Phosphorylation/De-phosphorylation

Smads can also be phosphorylated by other kinases at distinct sites, which allows different signaling pathways to both modulate Smad activity and impinge on the TGF-β-regulated transcription. The R-Smads and Smad4 are phosphorylated at proline-directed serine and threonine residues by ERK1/2, and by p38 and JNK. These phosphorylation sites are localized predominantly within the linker region of the Smads, but have also been reported in the MH1 domains of Smad2. Smad2 is phosphorylated at serine and threonine residues within its linker domain by mitogens such as EGF and HGF. In addition, MEK kinase 1 (MEKK1), an upstream activator of p38 and JNK, has been reported to stimulate linker phosphorylation of Smad2 and Smad3. A number of molecular mechanisms have been suggested to explain the increase in transcriptional activity of linker phosphorylated Smad2 and Smad3, including enhanced stabilization of nuclear Smads and stimulation of interactions with Smad4 or co-activators such as p300. In contrast, RAS stimulated activity of ERK1/2 induces phosphorylation of Smad2 and Smad3 within their linkers and inhibits their transcriptional activity and nuclear accumulation. Furthermore, Cyclin-dependant kinases CDK2 and CDK4, which govern the G1 to S phase transition of the cell cycle, also phosphorylate Smad2 and Smad3 at several sites within the MH1 and linker region of the proteins. CDK2 and CDK4 reduce Smad3 transcriptional and antiproliferative functions (Ross and Hill 2008). Linker Phosphorylation of Smad2/3 induced by TGF-β, RAS, PDGF and HGF through JNK induces Smad accumulation in the nucleus, suppresses their C-terminal phosphorylation by TβRI, and activates their transcriptional activity upregulating the expression of c-Myc, PAI-1, MMP-1, MMP-2, and MMP-9, resulting in strongly enhanced tumor growth and invasion (Matsuzaki and Okazaki 2006; Sekimoto et al. 2007). TGF-β induced JNK phosphorylation of Smad3 linker promotes carcinogenesis in human chronic liver disorders (Nagata et al. 2009).

Many phosphatases have now been described that can remove phosphate groups from both the C-terminus and linker residues of the R-Smads; for instance, pyruvate dehydrogenase phosphatase (PDP) and RNA polymerase II small C-terminal phosphatases SCP1, SCP2 and SCP3. PPM1A was identified as a nuclear R-Smad phosphatase that directly dephosphorylates C-terminal phosphorylated Smad1, 2 and 3 limiting the activation state of Smad2/3 and promoting their nuclear export (Itoh and ten Dijke 2007; Ross and Hill 2008). Smad7 recruits a phosphatase complex of GADD34 and PP1c, serine–threonine protein phosphatase 1, to the activated TGF-β type I receptor. SARA enhanced the recruitment of PP1c to TβRI and the dephosphorylation of TβRI (Itoh and ten Dijke 2007; Kang et al. 2009).

Sumoylation

Small ubiquitin-like modifier (SUMO), an ubiquitin related polypeptide, has also been shown to be important in regulating gene expression through altering the activity of target proteins. SUMO-1 is a 98 amino acid polypeptide that is covalently attached to the lysine residues of substrate proteins via a mechanism analogous to ubiquitination, and has been described to regulate transcription factor activity in several ways. First, sumoylation competes with other regulatory post-translational modifications such as acetylation and ubiquitination for lysine residues. In addition, SUMO modification can alter the affinity of a protein for an interaction partner. For example, several SUMO-modified transcription factors have increased binding affinity for histone deacetylases (HDACs) (Gill 2005). Protein inhibitors of activated STAT (PIAS) proteins, which have SUMO E3 ligase activity, have also been implicated in the regulation of Smad-dependent gene expression. PIASy is able to interact with Smad1, Smad2, Smad3 and Smad4, these interactions result in inhibition of TGF-β-induced activity in some, but not all, Smad3/Smad4-dependent promoters. Other members of the PIAS family, PIAS3 and hZimp10 can enhance Smad3/Smad4 transcription. Post-translational modification of Smad4 by sumoylation appears to modulate its transcriptional function. TβRI is also modified by SUMO groups after TGF-β stimulation. TβRI sumoylation is dependent on the kinase activity of the receptor and enhances its function by facilitating the recruitment and phosphorylation of Smad3 (Ross and Hill 2008; Heldin et al. 2009; Kang et al. 2009).

Acetylation

Acetylation is another lysine-specific post-translational modification that regulates the functions of numerous cellular proteins, most famously histones, but also non-histone proteins such as transcriptional regulators. Transcription factor acetylation can have different consequences, such as affecting the mechanism of DNA-binding and co-factor interactions. Smad7 is a substrate of acetylation by the HAT activity of the co-activator p300, and is acetylated at lysine residues K64 and K70. Several class I and class II HDACs can interact with Smad7 and mediate its deacetylation, an enhanced HDAC activity correlates with an increase in Smad7 ubiquitination and turnover. SIRT1, a class III HDAC, can also deacetylate Smad7 and enhance its Smurf-mediated ubiquitination and degradation. R-Smads are also acetylated by p300 and CBP. Acetylation of lysines within Smad2 and Smad3 enhances their transcriptional activity, and increases the DNA-binding efficiency of Smad3. In addition, Smad2 acetylation regulates nuclear localization possibly through decreasing the rate of nuclear export (Ross and Hill 2008).

TβR Internalization

At the plasma membrane, TGF-β receptors are also found in lipid rafts, dynamic micro-domains of proteins and lipids that are enriched in cholesterol and sphingolipids, as well as in non-lipid raft regions of the membrane. This differential localization at the cell surface might regulate their signaling function. TGF-β receptors can be internalized via at least two distinct internalization routes that predetermine whether receptors induce a signaling response or receptor degradation. Clathrin-dependent internalization of TGF-β receptors promotes signaling by guiding the receptor to early endosomes that are enriched with SARA. Via this route, the receptors can be recycled back to the cell surface. Inhibition of clathrin-mediated endocytosis decreases or prevents TGF-β-induced Smad activation. Internalization of TGF-β receptors via lipid-raft–caveolae-1 vesicles that contain receptors bound to I-Smad/Smurf targets the receptor for polyubiquitination and degradation. The I-Smad/Smurf complex is first formed in the nucleus and is subsequently targeted to lipid raft vesicles. Upon association of the Smad7/Smurf2 complex with an active TGF-β receptor, both Smad7 and the

receptor are ubiquitinated and destined for proteasomal and lysosomal degradation. However, signaling activation and receptor degradation have also been described to be associated with clathrin-mediated endocytosis (Itoh and ten Dijke 2007; Kang et al. 2009).

6.2. TGF-β biological roles

6.2.1. TGF-β and Growth inhibition

TGF-β induces cell cycle arrest in epithelial, endothelial, neuronal and hematopoietic cells at the early G1 phase via Smad-mediated transcriptional regulation of critical regulators of the cell cycle. TGF-β inhibits cell cycle progression through the induction of cyclin-dependent kinase (CDK) inhibitors and the transcriptional repression of c-myc, and ID1-3 genes (Pardali and Moustakas 2007; Massague 2008). TGF-β induces the expression of p15INK4b and p21CIP1 (Hannon and Beach 1994; Polyak et al. 1994; Datto et al. 1995). p15INK4 binds to CDK4 and CDK6, displacing p27KIP1 and p21CIP1, which can then associate with available CDK2 and inhibit its activity (Reynisdottir et al. 1995; Reynisdottir and Massague 1997). In hematopoietic cells, TGF-β also induces the expression of p57KIP2, which has similar roles than p21CIP1 and p27KIP1 (Scandura et al. 2004). TGF-β can also inhibit translation of CDK4 mRNA through a p53 dependent-manner (Ewen et al. 1995). Inhibition of the various G1 CDK complexes prevents the hyperphosphorylation of pRB and other substrates, so genes essential for S phase progression are not transactivated (Laiho et al. 1990). TGF-β also represses the expression of the Cdc25A tyrosine phosphatase which is responsible of de-phosphorylating and activating G1 phase CDKs (Iavarone and Massague 1997; Nagahara et al. 1999). Furthermore, TGF-β induces the repression of critical factors that promote cell growth and proliferation. Transcriptional repression of ID1, ID2 and ID3 is mediated by Smad signaling (Siegel et al. 2003b; Kowanetz et al. 2004). IDs expression downregulation is necessary for TGF-β-induced growth inhibition, since ID2 antagonizes p21CIP1 antiproliferative effects, promoting proliferation (Pardali et al. 2005). C-myc repression is triggered by a complex consisting of Smad3, Smad4, E2F4/5 and the transcriptional repressor p107 (Chen et al. 2002). C-myc downregulation relieves the inhibition of p15INK4A and p21CIP1 transcription mediated by c-myc binding to their promoters (Seoane et al. 2001; Seoane et al. 2002). During TGF-β mediated cytostatic effects, Smads act in association with other factors, for instance Smads cooperate with the transcription factor Runx3 to promote p21CIP1 expression (Chi et al. 2005), Smad3/4 complexes with FOXO transcription factors and Sp1 to up-regulate p15INK4A and p21CIP1 expression (Seoane et al. 2004; Gomis et al. 2006). Other non-smad pathways induced by TGF-β contribute to its effects on growth inhibition; for instance, TGF-β induces the activation of JNK and p38 which stabilize p21 protein increasing its half-life (Kim et al. 2002b), the PP2A/p70S6K also mediate TGF-β G1 cell cycle arrest (Petritsch et al. 2000).

TGF-β is known to induce cell cycle arrest in hepatocytes at low doses (Sanchez et al. 1996), and to counteract proliferative signals induced by EGF or Insulin (Carr et al. 1986; Sanchez et al. 1998). In addition, the antiproliferative action of TGF-β is well documented in partial hepatectomy rat and mouse models, where TGF-β mRNA increases in the regenerating liver and reaches a peak after the major wave of hepatocyte cell division and mitosis have taken place in order to prevent uncontrolled hepatocyte proliferation (Braun et al. 1988; Mead and Fausto 1989).

6.2.2. TGF-β and apoptosis

TGF-β is a well known inducer of apoptosis. It modulates the expression of several genes related to the apoptotic machinery in a Smad dependent manner, but also regulates apoptosis through the activation of stress activated kinases among other pathways (Pardali and Moustakas 2007). Far from being well established, the apoptotic signaling induced by TGF-β is being studied in different laboratories and literature indicates that different mechanisms exist and a complex network of apoptosis-related events might occur, which would be dependent not only on the cell type, but also on the cell context and extracellular environment. It is worthy to note that, as it will be explained along the text, TGF-β might induce both pro- and anti-apoptotic signals even in the same cell.

Some reports relate the apoptosis induced by TGF-β with activation of death receptors. Apoptosis of human gastric SNU-620 carcinoma cells induced by TGF-β takes place through caspase-8-mediated Bid cleavage that triggers mitochondrial apoptotic pathway. These events are caused by the Fas death pathway in a Fas ligand-independent manner (Kim et al. 2004a). The adaptor protein DAXX has been shown to be required for TGF-β-induced apoptosis through the activation of JNK in epithelial cells and hepatocytes, DAXX is thought to physically associate with TβRII, and it is involved directly in TGF-β apoptotic signaling pathway (Perlman et al. 2001; Padua and Massague 2009). However, TGF-β can also play a protective role towards FasL-induced apoptosis by increasing FLIP expression that will inhibit caspase-8 and caspase-3 activation in microglia (Schlapbach et al. 2000) and in pancreatic β cells (Maedler et al. 2002).

TGF-β induces JNK and p38 activation through the upstream kinase TAK1 (Sorrentino et al. 2008), leading to apoptosis. However, TGF-β-induced activation of TAK1 can also promote activation of IKK, and consequently a transient activation of NF-κB (Arsura et al. 2003), known to up-regulate the expression of different anti-apoptotic genes, such as BCL-XL and IAPs. Another regulator of TGF-β-induced apoptosis is Smad7, that even though its main function is to inhibit TGF-β signaling, it has been reported to have a pro-apoptotic role, as it can inhibit NF-κB, thereby amplifying the apoptotic signal. In addition, Smad7 acts as a scaffolding protein to facilitate the activation of p38 interacting with TAK1 (Edlund et al. 2003), promoting apoptosis. Another pathway of activation of p38 is through the induction of GADD45b expression by TGF-β, that then interacts with MKK4, which activates p38, leading to caspase and BAD activation (Yoo et al. 2003). Activation of p38 MAPK was shown to be essential to TGF-β-induced apoptosis in mammary epithelial cells (Yu et al. 2002), and B cells (Schrantz et al. 2001), but not in hepatocytes (Herrera et al. 2001c). As mentioned above, TGF-β induces activation of JNK pathway, which has also been shown to be involved in TGF-β-induced apoptosis through the induction of AP-1 in a murine myeloid cell line M1 and in the human hepatocarcinoma cell line Hep3B (Yamamura et al. 2000). Moreover, JNK will phosphorylate Jun family members that will cooperate with Smads proteins in the regulation of apoptosis-related gene expression (Hofmann et al. 2003). However, role of JNK in TGF-β-induced apoptosis is controversial and, at least in the case of hepatocytes (Carmona-Cuenca et al. 2006), it might not play a relevant role.

Additionally, different studies have reported that TGF-β induces the production of ROS coincident with an apoptotic process in epithelial cells such as fetal rat hepatocytes (Sanchez et al. 1996), in lung fibroblasts (Thannickal and Fanburg 1995), in lens epithelial cells (Yao et al. 2007) and in leukemic cells (Motyl et al. 1998). This process requires *de novo* synthesis of proteins (Sanchez et al. 1997). Two different mechanisms might be involved in the early ROS production: first, an inducible NADPH oxidase system is responsible for the early increase of extramitochondrial ROS (Herrera et al. 2004);

secondly, TGF-β could increase ROS by depleting antioxidant proteins, such as catalase, MnSOD or γ-glutamylcisteine synthetase (Franklin et al. 2003; Herrera et al. 2004), and glutathione synthetase, transferase, reductase and peroxidase (Coyle et al. 2003). TGF-β-induced ROS production is necessary for the apoptotic process in hepatocytes (Sanchez et al. 1996), fibroblasts (Langer et al. 1996), or human lens epithelial cells (Yao et al. 2007); among other functions, ROS are required for an efficient mitochondrial-dependent execution of apoptosis (Herrera et al. 2001a; Herrera et al. 2001b).

Finally, TGF-β can modulate the expression of different members of the BCL-2 family. In regards to the anti-apoptotic members of the BCL-2 family, some reports show that TGF-β induces the down-regulation of BCL-2 (Francis et al. 2000) and BCL-XL (Herrera et al. 2001b; Spender et al. 2009); however, this is not a fact of matter, and other reports have provided information that shows that TGF-β enhances the expression BLC-XL (Prehn et al. 1996; Valdes et al. 2004), or MCL1 (Gingery et al. 2008). TGF-β also induces the expression of the pro-apoptotic proteins BMF and BIM, triggering the intrinsic apoptotic pathway (Ramjaun et al. 2007; Yu et al. 2008), other reports show that this cytokine promotes BAX (Teramoto et al. 1998) and BIK (Spender et al. 2009) expression. Additionally, TGF-β can down-regulate the expression of some IAP members such as survivin (Yang et al. 2008a), BIRC3/cIAP2/HIAP1 (Yu et al. 2008) and XIAP (Wang et al. 2008a) and also cleave them by caspase action to decrease their protein levels (Herrera et al. 2002). Interestingly, TGF-β-induced apoptosis can also be mediated by ARTS (Larisch et al. 2000), an unusual septin-like mitochondrial protein that binds and decreases the expression of XIAP counteracting the anti-apoptotic properties of the IAP family (Gottfried et al. 2004).

TGF-β can also induce anti-apoptotic signaling through the activation of AKT (Valdes et al. 2004; Wilkes et al. 2005; Song et al. 2006), which in turn can prevent TGF-β-induced apoptosis by different ways. For instance, AKT can phosphorylate Bad, preventing its association with BCL-XL, it can also prevent the transcriptional activation of Foxo1 (Valverde et al. 2004). Moreover, AKT can interact with Smad3 avoiding its translocation to the nucleus, therefore, blocking its ability to induce the expression of genes necessary for TGF-β-induced apoptosis (Conery et al. 2004). However, very often, AKT activation is transient and at later times after TGF-β treatment, phopho-AKT levels decrease (Valdes et al. 2004). This might be related to the fact that, in response to TGF-β, other genes are regulated that favour TGF-β suppressor arm, such as the lipid phosphatase SHIP expression, which is up-regulated and blocks PI3K-mediated phospholipid phosphorylation, therefore inhibiting AKT pathway (Valderrama-Carvajal et al. 2002) . Early activation of AKT might be related to the capacity of TGF-β to transactivate the c-SRC and the EGF Receptor pathway (Park et al. 2004; Murillo et al. 2005). Indeed, TGF-β would mediate autocrine production of EGF Receptor ligands, which confer resistance to its pro-apoptotic effects in hepatocytes (Del Castillo et al. 2006; Murillo et al. 2007). However, the capacity of hepatocytes to survive to TGF-β depends on cell differentiation (Sanchez et al. 1999). In this line of evidence, fetal rat hepatocytes respond to TGF-β inducing survival signals, whereas adult hepatocytes do not (Caja et al. 2007). The reason for these differences should be, at least, the low levels of AKT and the lack of TACE/ADAM17 activity (responsible for EGF Receptor ligands shedding) observed in adult hepatocytes.

The role of Smads in TGF-β-induced apoptosis is not yet completely established. However, different studies support a role for Smad3. Indeed, in hepatocytes Smad3 is required for both TGF-β induced cell cycle arrest and apoptosis (Ju et al. 2006; Yu et al. 2008) and Smad3 overexpression protects liver from HCC by promoting pro-apoptotic activity through TGF-β signaling and activation of p38 MAPK

(Yang et al. 2006). It has been suggested that in FaO rat hepatoma cells TGF-β induces apoptosis through the cleavage of BAD in a Smad3-dependent manner (Kim et al. 2002a). Finally, in hepatocytes it has been described that both Smad3 (Black et al. 2007) and Smad 4 (Ramjaun et al. 2007) might be required for TGF-β-induced apoptosis. Up-regulation of BIM and BMF expression are dependent on Smad4, p38 and the generation of ROS (Ramjaun et al. 2007).

6.2.3. TGF-β induces EMT

EMT in response to TGF-β is characterized phenotypically by downregulation of epithelial markers such as E-cadherin, specific keratins and ZO-1, and upregulation of mesenchymal markers such as fibronectin, FSP1, α-SMA and Vimentin. During EMT, the actin cytoskeleton becomes reorganized from a cortical arrangement to a stress-fiber network connected to focal adhesions (Pardali and Moustakas 2007).

TGF-β-induced EMT is driven by a transcriptional program that involves the zinc finger transcription factors Snail1 and Snail2, the ZEB family factors ZEB1 and ZEB2, and the basic helix–loop–helix (bHLH) factors E47, E2-2, and Twist (Peinado et al. 2004; Peinado et al. 2007). These repressors recognize E-box DNA sequences located near the transcriptional initiation site of the E-cadherin gene blocking its expression. As recently reviewed in Pardali and Moustakas (2007), in response to TGF-β, Smad signaling mediates EMT presumably by regulation of critical gene targets that may act as effectors of EMT, or that may be required for the maintenance and full progression of EMT. In addition, non-Smad signaling effectors also have an impact on EMT, often by the establishment of crosstalks with the canonical Smad pathway. In carcinoma cells expressing an activated RAS oncogene, ERK1/2 and PI3K activities contribute to the establishment of EMT. Moreover, NF-κB signaling cooperates with Smads and contributes to EMT in vitro and to metastasis in vivo in RAS-transformed breast carcinomas.

TGF-β induces the increase of the nuclear factor HMGA2 expression through Smad3/4-dependent mechanism, HMGA2 binds to Smads to regulate Snail1 and Twist expression during mammary EMT (Thuault et al. 2008). Similar to HMGA2, c-Myc binds to Smads and induces Snail1 expression in response to TGF-β (Smith et al. 2009). TGF-β induces several genes that encode major constituents of the extracellular matrix and matrix regulatory enzymes, including plasminogen activator inhibitor 1 (PAI-1), collagenase I and the collagens. Smads regulate PAI-1 expression in cooperation with transcription factors, Sp1 (Datta et al. 2000) and TFE3 (Hua et al. 1998), and other signals from ERK and Rac1 (Mucsi et al. 1996; Kutz et al. 2001). Fibronectin gene expression is regulated by Smads and JNK (Itoh et al. 2003), and RAS/MKK4/JNK1 pathway is responsible for the regulation of the urokinase-type plasminogen activator receptor gene by TGF-β (Yue et al. 2004), some of these changes are regulated directly by Snail (Olmeda et al. 2007). TGF-β signaling also induces the expression of ZEB proteins during EMT, which induces E-cadherin downregulation and promotes cell migration independently of the Snail transcription factors in mouse mammary epithelial cells (Eger et al. 2005). In addition, expression of Twist1 or Twist2 decreases E-cadherin, occludin and claudin-7 expression, increases vimentin and N-cadherin expression, and enhances migration and invasion (Yang et al. 2004). Interestingly, it was recently described that EMT inducing factors such as Twist, Snail, and TGF-β may also promote the expression of cell surface markers of presumptive tumor-propagating cells, also referred to as “cancer stem cells” (Mani et al. 2008). In another study, TGF-β concomitantly promotes EMT and the CD44+/CD24+ to CD44+/CD24- transition in CD24+ HMLER

cells, CD24- is associated with both human breast CSCs and normal mammary epithelial stem cells (Morel et al. 2008).

Loss of Smad3 in mice blocks EMT in response to the injury of the lens, retina, and kidney in vivo or by exposure to exogenous TGF-β in organ culture. Furthermore, Smad3 can directly or indirectly regulate transcription of a number of other genes involved in the EMT process, including epithelial and mesenchymal markers, extracellular matrix/cytoskeleton proteins, inhibitors of differentiation, and components of the Notch signaling pathway (Millet and Zhang 2007). In addition, keratinocytes derived from Smad3–/– mice show reduced migration in response to TGF-β (Ashcroft et al. 1999). In contrast, loss of Smad2 in hepatocytes or keratinocytes promotes EMT, as these cells appear mesenchymal and migrate faster than wild type cells (Ju et al. 2006; Hoot et al. 2008). The inhibitory Smads function as negative regulators of, and consequently repress, TGF-β-induced EMT. Smad6 controls the timing and the extent of EMT during cardiac valve formation (Desgrosellier et al. 2005), while increased expression of Smad7 blocks TGF-β induced EMT in multiple tissues including the liver (Dooley et al. 2008).

TGF-β, via the Smad pathway, induces expression of the ligand of Notch signaling, Jagged1 (Zavadil et al. 2001). Then, TGF-β cooperates with active Notch to regulate expression of the Hey1 transcriptional repressor during the establishment of EMT (Zavadil et al. 2004). Recent evidence demonstrated that Notch receptor signaling can also directly regulate Snail1 and Snail2/Slug (Leong et al. 2007). Furthermore, the TGF-β/Smad pathway also cooperates with RAS and Wnt signaling in inducing Snail expression in development and in tumor metastasis (Barrallo-Gimeno and Nieto 2005). Moreover, TGF-β induces expression of PDGF ligands and receptors, thus activating autocrine PDGF signaling that promotes EMT and in vivo metastasis of mammary epithelial cells and hepatocellular carcinomas (Gotzmann et al. 2006; Jechlinger et al. 2006).

TGF-β induces cellular motility through the activation of Rho GTPases as explained in the previous section, which also contributes to tight junction disassembly (Ozdamar et al. 2005). Additionally, TGF-β induces expression of several tropomyosin genes in a Smad- and p38 MAPK-dependent manner, which contributes to cytoskeletal contractility and metastatic carcinoma cell motility (Bakin et al. 2004). Induction of the transcription factor CUTL1 by TGF-β leads to activation of many genes that regulate cell motility, tumor cell invasiveness and extracellular matrix deposition (Michl et al. 2005). TGF-β also induces expression of α3β1-integrin in hepatocellular carcinoma cells and motility and invasiveness of these cells depend critically on the level of this integrin receptor (Giannelli et al. 2002). TGF-β can also activate SRC and FAK kinase signaling that also contribute to the EMT and invasiveness response of hepatocytes (Cicchini et al. 2008). JNK activation in response to TGF-β also seems to be crucial for TGF-β-induced EMT and cell migration, as chemical inhibition of JNK blocks TGF-β-induced increase in fibronectin, vimentin and α-SMA expression (Santibanez 2006; Liu et al. 2008).

In the last few years, different studies have described that TGF-β induces an EMT process in hepatocytes and in liver cancer cells (Sanchez et al. 1999; Rossmanith and Schulte-Hermann 2001). In this regard, TGF-β is known to trigger the epithelial–mesenchymal transition through up-regulation of Snail and Slug in physiological and pathological conditions (Spagnoli et al. 2000; Gotzmann et al. 2002; Valdes et al. 2002; Sugimachi et al. 2003; Miyoshi et al. 2005). EMT induced by TGF-β in hepatocytes induces cell dedifferentiation (Sanchez et al. 1999; Valdes et al. 2002). In this line of evidence, Snail represses HNF-4α, resulting in loss of epithelial markers and expression of mesenchymal proteins (Cicchini et al. 2006). Worthy, recent results have suggested the capacity of

TGF-β to transdifferentiate fetal hepatocytes to liver progenitors with capability of differentiating to both mature hepatocytes and cholangiocytes (del Castillo et al. 2008).

It is worthy to note that very often pro-apoptotic and EMT signals occur simultaneously in response to TGF-β, as is the case of fetal rat hepatocytes. Interestingly, the success of the EMT process initially depends on the capacity of cells to impair TGF-β-induced cell death. However, later on the EMT process induces survival signals that rescue cells from further suffering apoptosis (Valdes et al. 2002). Remarkably, as commented above, Snail over-expression protects cells from apoptosis (Vega et al. 2004). EMT might confer resistance to TGF-β suppressor effects through the increase in the expression of EGFR ligands and activation of the EGF Receptor pathway, which again points out to a clear crosstalk between TGF-β and EGF signals in hepatocytes (Del Castillo et al. 2006).

6.2.4. TGF-β and cancer

There have been many reports and reviews in which they described that the over-activity of TGF-β members is linked to various diseases, including fibrosis and malignancies. Under pressure to avoid tumor-suppressive effects, some cancer cells accumulate inactivating mutations in the TGF-β receptors and the Smad proteins. In normal, unstressed tissue, sustained basal release of TGF-β by local sources may be sufficient for the maintenance of homeostasis. However, under conditions of tissue injury, TGF-β is abundantly released; sources of TGF-β in tumors vary and include the cancer cells themselves, various cells of the tumor stroma, blood platelets as well as tumor-infiltrating cells (Massague 2008). Tumors are infiltrated by leukocytes, macrophages, and bone marrow-derived endothelial, mesenchymal, and myeloid precursor cells. TGF-β is known to act as a tumor suppressor in early stages of tumorigenesis, but it can also promote advanced tumor cell invasiveness and metastasis. As mentioned before, TGF-β is a potent anti-tumor agent because it strongly inhibits the growth of epithelial cells. However, in a different cellular context, TGF-β can also promote tumor growth because it is able to induce changes in transcriptional activities that re-program epithelial cells into mesenchymal cells, thereby facilitating tumor metastasis and invasion (Zhang 2009). TGF-β indirectly promotes self-sufficiency to growth signals, as it is over-produced by cancer cells and induces production of several mitogenic factors such as HGF or PDGF in the tumor stroma. TGF-β signals to the tumor stromal environment, by targeting fibroblasts, myofibroblasts, the vasculature and surveilling immune cells, thus promoting tumor cell survival, invasiveness, angiogenesis and metastasis in advanced cancer stages (Pardali and Moustakas 2007; Heldin et al. 2009). TGF-β is also a modulator of tumor microenvironment. This microenvironment, in addition to the extracellular matrix components is composed of cancer-associated fibroblast or myofibroblasts, immune cells and microvessels, and provides control of tumor growth (Pardali and Moustakas 2007).

While the various mechanisms of genetic and epigenetic inactivation of TGF-β signaling components explain the suppression of anti-tumoral action of this pathway in certain types of cancer, the large majority of tumors do not exhibit any of these alterations. Yet, most tumor cells exhibit relative resistance to TGF-β signaling in terms of growth inhibition or apoptosis. Such tumor cells may have a fully functional TGF-β signaling pathway, but specific regulatory events in the transmission of the signal may have been altered (Pardali and Moustakas 2007).

During cancer progression, high levels of TGF-β can promote tumor growth in an autocrine and/or paracrine manner through the changes that favor invasion and metastasis. Smad4 mutation and/or reduced expression correlate with poor prognosis and increased metastasis in colorectal cancer

(Miyaki et al. 1999), whereas Smad7 expression is associated with poor outcome in gastric carcinomas (Kim et al. 2004b). In contrast, Smad activation by TGF-β receptor is also required for the pro-metastatic function of TGF-β, and dominant negative Smad3 inhibits lung metastasis of breast cancer cells in animal models. In addition, blockade of Smad pathway by overexpression of the inhibitory Smad, Smad7, impairs bone and lung metastases (Pardali and Moustakas 2007; Halder et al. 2008). However, TGF-β signaling has shown disparate effects on metastasis in mouse models. Accordingly, active TβRI inhibits primary mammary cancer formation but it accelerates the rate of metastasis to lung (Siegel et al. 2003a; Muraoka-Cook et al. 2006). There is also evidence that reduced TβRII activation may selectively facilitate pro-oncogenic effects on the tumor cell at the expense of growth inhibition (Wakefield and Roberts 2002), it has been observed that tissue-specific inactivation of TβRII alone in mouse models leads to spontaneous tumor formation (Massague 2008). Genetic inactivation of TβRII, Smad2, and Smad4 in hepatocellular carcinoma has been reported (Furuta et al. 1999; Kawate et al. 1999; Yakicier et al. 1999), with various frequencies. It has been described that the loss of Smad2 function during carcinogenesis may increase the proliferative and metastatic potential, whereas loss of Smad3 function may decrease the metastatic potential of tumor cells (Ju et al. 2006). In addition, hepatocellular carcinomas, as well as breast cancer and lung carcinomas, also overproduce TGF-β in vivo and the higher the level of this cytokine is, the higher the degree of neo-vascularization and their chance for metastasis (Pardali and Moustakas 2007).

Other signaling pathways can counteract TGF-β-induced suppressor effects. Aberrant activation of MAPK pathways may play an important role in promoting the TGF-β response towards a pro-oncogenic outcome. Activation of Raf confers protection against TGF-β-induced apoptosis while enhancing its pro-invasive effects. EGFR signaling through the RAS/MEK pathway blocks Smad activity. In addition, Protein kinase C modifies the DNA binding of Smad3 and impairs the growth inhibitory and pro-apoptotic actions of TGF-β (Wakefield and Roberts 2002).

Interestingly, it has recently been reported that TGF-β can induce stemness independently of an EMT process. TGF-β induces the self-renewal capacity of patient-derived gliomas-initiating cells and prevents these cells differentiation through the induction of LIF (a known inducer of mouse embryonic stem cell self-renewal) expression, which in turn promotes the activation of JAK/STAT pathway. Together these results suggest that TGF-β may have an important role in maintaining the stem cell-like pool in gliomas (Penuelas et al. 2009). It has been also described that TGF-β allows the retention of stemmnes properties of glioma-initiating cells through up-regulation of Sox2, a stemness gene, and this induction is mediated by Sox4, a direct TGF-β target gene (Ikushima et al. 2009). Moreover, TGF-β treatment increases the percentage of Side Population cells in hepatoma cells, phenomena related to the appearance of cells with stem cell properties and resistance to therapeutic drugs (Nishimura et al. 2009).

The ability of tumor cells to induce new blood-vessel formation from pre-existing vasculature is essential for the progressive growth and dissemination of tumor cells. TGF-β can induce the expression of angiogenic factors such as VEGF and connective-tissue growth factor (CTGF) in both epithelial cells and fibroblasts. Angiopoietin-1 (ANG1) a ligand for the TIE2 receptor tyrosine kinase maintains vessel integrity and is suppressed by TGF-β in fibroblast cells. The ability of the tumor to recruit endothelial cells requires the dissolution of mature vessels in the vicinity of the tumor. TGF-β induces the expression, secretion and activity of matrix metalloproteinases, such as MMP2 and MMP9, and downregulates the expression of the protease inhibitor TIMP (Tissue Inhibitors of Metalloprotease) in both endothelial cells and tumor cells, allowing release of the endothelial cells

from the basement membrane. So, TGF-β can create a pro-angiogenic environment (Siegel and Massague 2003; Pardali and Moustakas 2007; Padua and Massague 2009).

A final and very important function of TGF-β in the tumor stroma is the modulation of immune cells. TGF-β-mediated T-cell immunosuppression is of special relevance during tumor progression because tumor-derived TGF-β is believed to impair tumor recognition and clearance by cytotoxic T lymphocyte (CTLs). TGF-β regulates T-cell function through several mechanisms including prevention of interleukin-2 (IL-2) production and subsequent T-cell activation, induction of peripheral T-cell apoptosis, and inhibition of CTL, T-helper 1 (TH1) and T-helper 2 (TH2) differentiation, resulting in the most potent natural immunosuppressor in the human body (Siegel and Massague 2003; Pardali and Moustakas 2007). TGF-β-mediated inhibition in the production of several cytokines, such as IL-2, IL-13, IL-15, and IFN- γ expression was lost in the Smad3-deficient primary T-cell cultures (Millet and Zhang 2007). Similarly, TGF-β signaling inhibits expression of perforin, granzyme A and B, and Fas ligand which act together in order to elicit cytotoxicity mediated by CTLs. At least in the case of IFN-γ and granzyme B, Smad directly represses their transcription (Thomas and Massague 2005). In addition, TGF-β deactivates scavenging macrophages and thus protects the developing tumor from proper immune surveillance. If TGF-β is neutralized, the CTL cytotoxic program is restored and it mediates its anti-tumor action. TGF-β can also impair T-cell activation through negative effects on antigen-presenting cells (APCs) such as dendritic cells. During an immune response, dendritic cells mature and acquire the ability to effectively stimulate T cells; this process is blocked by TGF-β (Geissmann et al. 1999). Moreover, TGF-β can inhibit the expression of major histocompatibility complex class II molecules on various cells by inhibiting the expression of the class II transactivator through a Smad3-dependent mechanism. Additional targets of TGF-β-mediated immunosuppression in tumorigenesis might include natural killer cells and neutrophils (Siegel and Massague 2003). In contrast, TGF-β-mediated immunosuppression may contribute to its tumor-suppressing role by inhibiting inflammatory responses upon bacterial infections that predispose animals to cancer formation as documented in Smad3-deficient mice (Maggio-Price et al. 2006).

7. EGFR

The ERBB family of proteins (originally named because of their homology to the erythroblastoma viral gene product, *v-erbB*) comprises four receptors (ERBB1–4, also known as HER1–4) and 13 polypeptide extra cellular ligands, which contain a conserved epidermal growth factor (EGF) domain (Citri and Yarden 2006). Intracellular pathways activated by the EGFR show a high degree of interaction and control different transcriptional programs that regulate the expression of genes involved in cell-cycle progression, survival, differentiation, and cell migration.

Both EGF ligands and ERBB receptors are produced as glycosylated transmembrane proteins. The receptors are composed of a large extracellular ligand-binding domain, which has four subdomains (I–IV), followed by a transmembrane domain, a small intracellular juxtamembrane domain preceding the kinase domain, and a C-terminal tail, on which the docking sites for phosphotyrosine-binding effector molecules are found. Of the four subdomains in the extracellular region of the ERBB receptors, subdomains I and III are leucine-rich repeats that function in ligand binding (also called L1 and L2), whereas subdomains II and IV are laminin-like, cysteine-rich domains (also called CR1 and CR2) (Citri and Yarden 2006). The subdomains I, II and III from the extracellular domain form a ligand-binding pocket, where a ligand is docked between domains I and III (Zandi et al. 2007). The juxtamembrane region appears to have a number of regulatory functions, i.e., downregulation and ligand dependent internalization events, basolateral sorting of the EGFR in polarized cells, and association with proteins such as eps8 and calmodulin. The carboxy-terminal domain of the EGFR contains tyrosine residues where phosphorylation modulates EGFR mediated signal transduction. There are also several serine/threonine residues where phosphorylation has been inferred to be important for the receptor downregulation processes and for endocytosis. ERBB1 also interacts with its three known homologues, ERBB2, ERBB3, and ERBB4 to form heterodimers. Differences in the C-terminal domains of these proteins results in changes to the repertoire of signaling molecules that interact with the heterodimers, thus leading to an expansion in the number of possible signaling pathways stimulated by a single ligand (Jorissen et al. 2003).

Two members of the family, ERBB2 (also known as HER2/neu) and ERBB3, are non-autonomous. ERBB2 lacks the capacity to interact with a growth-factor ligand, whereas the kinase activity of ERBB3 is defective. Despite this lack of autonomy, both ERBB2 and ERBB3 form heterodimeric complexes with other ERBB receptors that are capable of generating potent cellular signals. The autonomous receptor **ERBB1** binds to multiple ligands and forms homodimers, as well as three functional heterodimers. Several tyrosine-based motifs recruit a number of signal transducers to the phosphorylated form of ERBB1 in order to activate a broad number of signaling pathways. **ERBB2** is a non-autonomous amplifier of the network, rather than an additional growth-factor receptor. ERBB2 incapable of binding to a ligand due to the strong interaction between domains I and III that leads to a constitutively extended dimerization arm. Although ERBB2 does not bind to EGF-like ligands, it functions as the preferred heterodimeric partner of the other three ERBB members. ERBB2 binds to a much larger subset of phosphotyrosine-binding proteins than the other ligand binding receptors of the family. ERBB2-containing heterodimers undergo slow endocytosis, and they recycle back to the cell surface more frequently. These features translate to potent mitogenic signals owing to the simultaneous and prolonged recruitment of multiple signaling pathways. As such, ERBB2 is constantly primed for interactions with ligand bound receptors of the family. **ERBB3** is a kinase-defective non autonomous receptor that binds to four ligands and forms three functional heterodimers. On heterodimerization, the cytoplasmic domain of ERBB3 undergoes tyrosine phosphorylation and can recruit PI3K to six distinct sites and Shc to one site, although there is no site for Grb2, ErBB3 is

considered the major PI3K activating ErBB receptor. **ERBB4**, the other autonomous receptor, shares recognition and signaling features with ERBB1. They both bind to a large and distinct group of ligands, the betacellulin and the heparin-binding ligand, HB-EGF, as well as to two low-affinity ligands, epiregulin and epigen. Like ERBB1, ERBB4 recruits Grb2, Shc and STAT5, whereas one isoform of ERBB4 (CYT-1) can activate PI3K (Citri and Yarden 2006; Hynes and MacDonald 2009).

7.1. EGFR ligands

The EGF-family of peptides binds the ERBB receptors and on the basis of their receptor specificity is divided into three groups. The first group includes EGF, TGF-α, amphiregulin (AR), and epigen (EPG), which bind specifically to EGFR/ERBB1; the second group includes betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR), which exhibit dual specificity, binding both EGFR and ERBB4. The third group, the neuregulins (NRGs), forms two subgroups on the basis of their capacity to bind ERBB3 and ERBB4 (NRG-1 and NRG-2) or only ERBB4 (NRG-3 and NRG-4) (Hynes and MacDonald 2009). Each of the mature peptide growth factors is characterized by a consensus sequence consisting of six spatially conserved cysteine residues (CX7 CX4–5 CX10–13 CXCX8 C) that form three intramolecular disulfide bonds. This consensus sequence is known as the EGF motif, an immunoglobulin-like domain, which is crucial for binding members of the ERBB receptor tyrosine kinase family, there are additional motifs such as glycosylation sites and heparin-binding domains in AR and HB-EGF (Harris et al. 2003; Citri and Yarden 2006; Berasain et al. 2009). Knockout of specific ERBB1 ligands indicated that there is a significant functional redundancy between the ligands. No significant phenotype was observed in the knockouts of the ERBB1 ligands EGF, AR or TGF-α-deficient mice which only had eye abnormalities and derangement of hair follicles. Abnormalities in the small intestine were only observed in the EGF, amphiregulin and TGF-α triple knockout (Mann et al. 1993; Luetteke et al. 1999; Troyer et al. 2001).

The membrane-anchored peptide can be biologically active through juxtacrine signaling, in most cases the extracellular domain is proteolytically cleaved by a metalloprotease activity present in the cell membrane. This process is known as ‘‘ectodomain shedding’’ and leads to the release of the soluble growth factor, which may act in an endocrine, paracrine, or autocrine fashion (Berasain et al. 2009). Epidermal growth factor (EGF) is a 53-aa polypeptide, with a molecular weight of 6045, that is derived by proteolytic processing from the transmembrane precursor (prepro-EGF) of 1207 aa in humans or 1217 aa in rodents (Harris et al. 2003).

Various members of the ADAM family, membrane-anchored metalloproteases of the ADAM family (a disintegrin and metalloprotease), have been implicated in EGFR ligand cleavage including ADAM 9, 10, 12, 15, 17, and 19. However, ADAM17, which is also known as tumor necrosis factor-α (TNF-α)-converting enzyme, or TACE, together with ADAM10, are thought to play a central role. The ADAM family of metalloprotease disintegrins has a modular transmembrane domain with a Zinc dependent catalytic domain (Borrell-Pages et al. 2003). ADAM17 can cleave the AR, TGF-α, and HB-EGF membrane anchored precursors, while ADAM10 is a key sheddase for EGF, BTC, and can also cleave the HB-EGF transmembrane precursor. The proteolytic activity of ADAMs is therefore crucial for the generation of soluble EGFR ligands and receptor activation. Importantly, the proteolytic activity of ADAMs is in turn subject to regulation by multiple upstream signals. In fact, there is a growing list of physiological stimuli that can trigger EGFR signaling through the stimulation of ligand shedding, a process known as EGFR transactivation. Transactivation of the EGFR by ligands of G protein-coupled receptors (GPCRs) is perhaps the best characterized example of EGFR

activation by heterologous ligands. These include angiotensin II (ANG II), lysophosphatidic acid (LPA), endothelin-I, thrombin, IL-8, and prostaglandins such as PGE2. Different mechanisms have been proposed to mediate ADAM activation by GPCRs. Transactivation of the EGFR is not exclusive of G protein-Coupled Recdeptors (GPCR)-triggered signaling; different cytokines can trigger ADAM17 activation. EGFR ligands can be elicited by the cytokines TNF-α, INF-γ and Fas ligand (FasL). Activation of different TLRs has been reported to promote wound repair and to trigger inflammatory signaling through EGFR transactivation (Berasain et al. 2009). In this case, ROS generated by the NADPH oxidase dual oxidase 1 were implicated in the activation of ADAM17, and in the initiation of innate immune responses in airway epithelial cells (Koff et al. 2008). TRAIL promotes ADAM17-mediated TGF-α shedding in a c-SRC-dependent fashion in colorectal cancer cells. Importantly, the transactivation of EGFR by TRAIL constitutes a mechanism of resistance towards TRAIL-induced apoptosis in these tumoral cells (Van Schaeybroeck et al. 2008). Furthermore, recent studies have shown that TGF-β induces pro-survival signaling through the activation of ERBBs receptors by inducing TACE activity (Murillo et al. 2005; Wang et al. 2008b; Wang et al. 2009a).

7.2. EGFR activation and signaling

Upon ligand-mediated activation, each receptor may form homo- or heterodimers and cross-phosphorylate each other (Ferguson et al. 2003). However, other reports have shown that ERBB receptors exist in a pre-dimerized state (Gadella and Jovin 1995; Sako et al. 2000). Ligand binding to this pre-dimerized state forms a 2:2 ligand to receptor configuration and induces a rearrangement of each receptor subunit. Ligand binding triggers ERBB autophosphorylation in distinct tyrosine residues, creating docking sites for several signaling proteins such as Shc, Grb7, Grb2, Crk, phospholipase Cc (PLCc), the kinases SRC and PI3K, the protein phosphatases SHP1 and SHP2, and the Cbl E3 ubiquitin ligase. There are other signaling proteins, like phospholipase D (PLD) and the STAT 1, 3, and 5 proteins that do not bind the ERBB receptors but are also activated upon ligand binding. The adaptor proteins growth-factor-receptor bound-2 (Grb2) and Src-homology-2-containing (Shc) are responsible for the recruitment of RAS and activation of the mitogen-activated protein kinase (MAPK) cascades. Both Grb2 and Shc play important roles in the activation of other EGFR-dependent pathways. This is due to their “modular” construction. Grb2 contains two SH2 domains and one SH3 domain, which enable it to interact with tyrosine-phosphorylated motifs as well as with proline-rich regions of other proteins. Shc can associate with specific tyrosine-phosphorylated sequences via its SH2 and PTB domain, and, being itself phosphorylated on tyrosine by activated receptors and cytosolic tyrosine kinases, serves in turn as a binding partner for SH2-containing proteins. These interactions trigger intracellular signaling pathways such as c-jun NH2-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38-MAPK), the protein kinase C (PKC) pathway, the PI3K/AKT pathway (which can lead to NF-κB activation), and the STAT pathway. ERBB1 cannot directly activate the PI3K/AKT-protein kinase B (PKB) pathway, but it couples to the RAS/MAPK pathway, as well as to the RAS/PI3K/AKT-PKB pathway. ERBB1 signaling is negatively regulated through ubiquitylation by Cbl. The ERBB network displays redundancy. For example, in the pathway that leads to activation of Raf1 by son of sevenless (SOS), ERBB1 can recruit SOS through either Grb2 or Shc, whereas Grb2 can associate with the receptor either directly or through Shc. Likewise, Cbl is recruited to ERBB1 either directly (Tyr1045), or indirectly, through Grb2 (Jorissen et al. 2003; Citri and Yarden 2006; Berasain et al. 2009). EGFR can also be

transphorylated and therefore activated by other kinases such as SRC and JAK2 (Yamauchi et al. 1997; Tice et al. 1999).

Since the main pathways activated by EGFR are RAS/Raf/MEK/ERK, STAT and PI3K a more detailed explanation is provided. In the case of RAS pathway, Grb2 is constitutively bound to the RAS exchange factor SOS and is normally localized to the cytosol. Following activation of the EGFR kinase and autophosphorylation, the SH2 domain of Grb2 can bind to the EGFR. Grb2 can associate with the receptor either directly (via Y1068 and Y1086) or indirectly, by binding to EGFR-associated, tyrosine phosphorylated Shc. Relocation of the Grb2/SOS complex to the receptor at the plasma membrane facilitates the interaction of membrane-associated RAS with son of sevenless (SOS), resulting in the exchange of RAS-bound GDP for GTP and hence in RAS activation. Activated RAS in turn activates the serine/threonine kinase Raf-1. Raf-1 activation, through a series of intermediate kinases, leads to the phosphorylation, activation, and nuclear translocation of ERK1 and ERK2, which catalyze the phosphorylation of nuclear transcription factors (Jorissen et al. 2003).

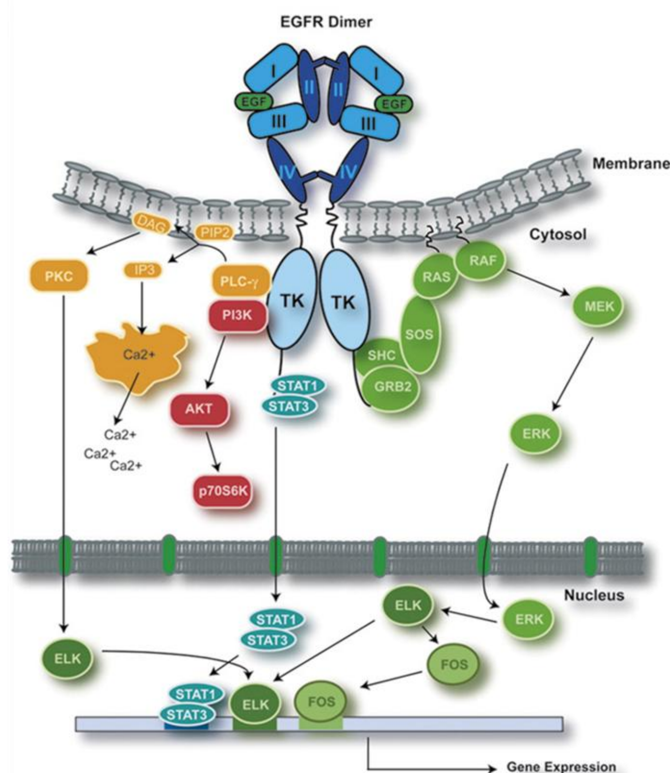


Figure 15. EGFR downstream signaling pathway. From Zandi et al., 2007.

In mammals, seven STAT genes have been identified (STAT 1 to 4, 5a, 5b, and STAT6). STAT proteins are inactive transcription factors, which are activated and translocated to the nucleus upon specific receptor stimulation. Classically, STATs are recruited to the intracellular domain of the cytokine receptors through specific binding between STAT SH2 domains and receptor phosphotyrosine residues. Homo- and hetero-dimerization of STAT proteins is a prerequisite for activation and translocation to the nucleus, and is mediated by tyrosine phosphorylation of critical residues. In cytokine signaling, activation is mediated by the JAK family of kinases. STAT proteins, in particular STAT-1, 3, and 5, have also been implicated in EGFR signaling. The ligand-dependent phosphorylation of STATs by EGFR does not require JAK kinases. STATs do not bind to the C-terminal phosphotyrosines of the EGFR; indeed it appears that STATs are constitutively associated with the EGFR (Jorissen et al. 2003).

Phosphoinositide-3-kinases are major players in cellular functions, where they contribute to a variety of cellular processes including proliferation, survival, adhesion, and migration. PI3-kinases catalyze phosphorylation on the 3’ position of phosphatidylinositols (PtdIns) and are assigned to three classes according to their subunit structure and their preferred lipid substrate. Of the three classes of typical PI3-kinases, only class Ia is activated by tyrosine kinase receptors. Interaction between PI3K and the

ERBB receptors is required for activation, and is mediated by association of the phosphorylated receptors with the p85 subunit of PI3K via the latter's SH2 domain. The major binding partner of p85 is not the EGFR, but ERBB3; however, activation of PI3K is observed in response to EGFR ligands through formation of ERBB1/ERBB3 heterodimers, as well as potentially by SRC phosphorylation of the EGFR. PI3K Ia generates phosphatidylinositol-3,4,5-trisphosphate (PIP3). One of the best characterized targets of this second messenger is the Ser/Thr kinase AKT (PKB), which binds to the lipid and is translocated to the plasma membrane where it is phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDK1) and possibly other kinases. PKB/AKT is a major mediator of PI3K action in survival and proliferation, and may well be the major mediator of the anti-apoptotic effects of EGFR activation (Jorissen et al. 2003).

There are evidences of the presence of ERBB receptors in the nucleus of highly proliferative cells where it functions as a transcription factor. Although EGFR has transactivational activity, it lacks a DNA binding domain and, therefore, may require a DNA binding transcription cofactor for its transcriptional function. For instance, ADAM17/TACE cleaves ERBB4 at the extracellular domain, whereas a γ-secretase activity cleaves it within the transmembrane domain. The second cleavage generates a soluble 80 kDa protein (s80) that translocates to the nucleus (Ni et al. 2001). s80 is a chaperone that facilitates the nuclear entry of the transcription factors STAT5 (Williams et al. 2004). Other ERBB members have been shown to have a transactivational function; ERBB1's is due to its physical association with STAT3 in the nucleus, which leads to activation of transcription, whereas ERBB2 directly binds to the COX2 promoter (Lo et al. 2005).

ERBB mediated activation of the RAS/MAPK pathway strongly induces the transcription of multiple ERBB ligands, including TGF-α and HB-EGF. Similarly, transactivation of ERBB1 by G-protein-coupled receptors occurs through the stimulation of surface proteinases, generating mature, active HB-EGF. Therefore, creating a positive feedback loop (Citri and Yarden 2006).

Inactivation of the EGFR can be mediated either by receptor dephosphorylation by phosphotyrosine phosphatases or receptor downregulation. Receptor downregulation is the most prominent regulator of EGFR signal attenuation and involves the internalization and subsequent degradation of the activated receptor in the lysosomes (Zandi et al. 2007). In general, ligand induced receptor endocytosis downregulates growth factor signaling. Nevertheless, accumulating evidence indicates that internalized receptors might couple to effectors in pre-degradative intracellular compartments, and thereby activate signaling pathways distinct from those that are activated at the cell surface. For example, internalized ERBB1 molecules are enzymatically active, hyperphosphorylated and associated with Shc, Grb2 and SOS. Another general mechanism of signal attenuation that functions at the receptor level is instigated by tyrosine phosphatases such as density-enhanced phosphatase-1 (DEP1), which dephosphorylates ERBB1 as well as other RTKs (Receptor Tyrosine Kinases), and protein tyrosine phosphatase-1B (PTP1B), which dephosphorylates RTKs in endosomes. EGF treatment induces expression of the suppressor of cytokine signaling-5 (SOCS5) that leads to a marked reduction in the levels of the receptor by promoting ERBB1 degradation, possibly by the 26S proteasome. Two other newly induced proteins that participate in ERBB homeostasis through Cbl are the adaptor protein sprouty (SPRY) and leucine-rich repeats and immunoglobulin-like domains-1 (LRIG1). SPRY2 regulates the RAS/MAPK pathway through several regulatory mechanisms, including inhibition at the levels of Grb2, RAS-GTPase activating protein (RAS-GAP), or the Raf1 kinase. The phosphorylated form of SPRY2 binds to and sequesters Cbl. Similarly, up-regulation of LRIG1 is followed by enhanced ubiquitylation and degradation of ERBB1 through a mechanism that involves recruitment of Cbl and simultaneous ubiquitylation of both ERBB1 and LRIG1 (Citri and Yarden 2006).

Hepatocytes of the mature liver express the highest levels of EGFR of any non-transformed cell, suggesting an important role for ERBB signaling in normal liver function (Dunn and Hubbard 1984), and when isolated hepatocytes are treated with EGFR ligands such as EGF, TGF-α, AR, HB-EGF, and EREG, a potent mitogenic (Fausto et al. 1995; Block et al. 1996; Mitchell et al. 2005) and anti-apoptotic (Fabregat et al. 1996; Fabregat et al. 2000) effect is observed. The hepatoprotective and pro-regenerative potential of the EGFR axis has been demonstrated in transgenic mice overexpressing TGF-α or HB-EGF, or by the direct intraperitoneal administration of these ligands in models of acute injury and regeneration (Berasain et al. 2007). EGFR ligands are known to be upregulated in the hepatic parenchyma during surgically induced regeneration and experimental tissue injury. Interestingly, it has been published recently that the expression of ADAM17 is also upregulated during liver regeneration after partial hepatectomy in the rat (Lin et al. 2008). The only ERBB proteins expressed in the adult rat liver are EGFR and ERBB3. Although ERBB2 is not expressed in the normal or regenerating adult liver or in primary hepatocytes derived from adult animals, it is expressed in the fetal and neonatal liver, in stem-like RLE 13 cells, and in the H4IIe hepatoma cell line (Carver et al. 2002).

7.3. EGFR in cancer

The involvement of increased and/or aberrant EGFR activity in human cancers is well documented and cancer patients with altered EGFR activity tend to have a more aggressive disease, associated with a poor clinical outcome. There are quite a few mechanisms by which the tight regulation of the EGFR-ligand system can be abrogated. These include: 1) increased production of ligands, 2) increased levels of EGFR protein, 3) EGFR mutations giving rise to constitutively active variants, 4) defective downregulation of EGFR and 5) cross-talk with heterologous receptor systems (Zandi et al. 2007; Hynes and MacDonald 2009).

Overexpression of the ligands and/or receptors, as well as ligand-independent receptor activation, occurs in many epithelial cancers, most notably gliomas and breast, pancreas, and liver carcinoma (Jorissen et al. 2003). Elevated levels of EGFR have been found to correlate with decreased survival in various cancer types including head and neck, bladder, ovarian, cervical and esophageal cancer. This transforming ability of overexpressed EGFR is likely due to constitutive receptor activation caused by spontaneous receptor dimerization as a result of high EGFR levels on the cell surface. A constitutively active receptor results in continuous activation of downstream signaling pathways, leading to a more malignant phenotype (Pedersen et al. 2004; Pedersen et al. 2005). Likewise, overexpression of ERBB2 has been reported in breast, lung, pancreas, colon, endometrium and ovarian cancer, and it has been associated with a poor prognosis for breast and ovarian cancer patients (Ross et al. 2003). In cancer patients, the autocrine production of TGF-α or EGF is associated with reduced survival (Tateishi et al. 1990; Hirai et al. 1998). In animal models, it appears that overexpression of TGF-α is linked to hyperproliferative responses but does not generally lead to tumors in rodents (Jorissen et al. 2003).

Even in transgene models where TGF-α-associated tumors are observed, the latency period tends to be long and the incidence low, suggesting that the TGF-α/EGFR system provides only one of the steps in multistage carcinogenesis, and neoplastic transformation only occurs when other genes within the target tissue are also mutated. Overexpression of c-myc in TGF-α transgenic mice dramatically

shortens the latency period and accelerates tumor growth (Murakami et al. 1993). There is cooperation between TGF-α and c-myc in the induction of hepatocarcinomas (Webber et al. 1994).

Mutations in the EGFR gene are frequent in human cancers. These mutations can be divided into three main groups: those that lead to changes in the extracellular domain, those that lead to changes in the intracellular domain and those that specifically lead to changes in the intracellular tyrosine kinase domain. Various mutations have been reported in the extracellular domain, usually due to large deletions or duplications of specific exons, and in almost all cases they arise in tumors with EGFR amplification. The most frequent, and by far best characterized, EGFR mutation is EGFRvIII. EGFRvIII is the result of an in-frame deletion of exons 2–7, which encodes the subdomain I. EGFRvIII is constitutively phosphorylated and able to activate tumor-promoting signaling pathways. Like EGFR, EGFRvIII is able to activate the downstream effector pathways RAS/RAF/MEK/ERK and PI3K/AKT. However, in contrast to ligand-activated EGFR, it has been demonstrated that EGFRvIII also activates the Jun N-terminal Kinase (JNK) via PI3K, but is unable to activate STAT1 and STAT3. EGFRvIII appears to be trapped in a partially activated state, which is sufficient for activation of oncogenic signaling pathways, but insufficient for receptor degradation, as the receptor fails to be ubiquitylated, thus prolonging the oncogenic signaling. As with the mutations in the extracellular domain, intracellular mutations primarily consist of either large deletions and/or duplications of exons. The last group of the EGFR mutations consists of small mutations that lead to changes in the intracellular tyrosine kinase domain. These small mutations prolong the tyrosine kinase activity of ligand-activated receptors (Zandi et al. 2007).

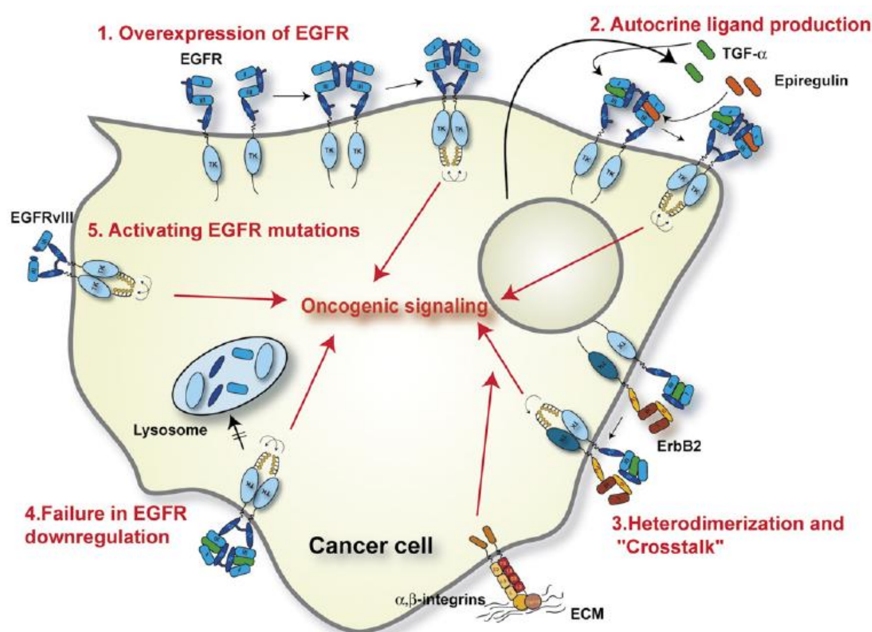


Figure 16. Mechanisms leading to EGFR oncogenic signaling. From Zandi et al., 2007.

EGFR downregulation is a mechanism by which EGFR signaling is attenuated and it involves the internalization and subsequent degradation of the activated receptor. As mentioned above, aberrant EGFR signaling due to defective receptor downregulation has also been linked to neoplastic cell transformation. One of the mechanisms of aberrant EGFR downregulaton, is the lack of c-Cbl binding site in EGFR, c-Cbl is an ubiquitin ligase that plays a central role in EGFR downregulation. Another mechanism is through by overexpression of ERBB2. Unlike ligand-activated EGFR, activated ERBB2 is not downregulated and when expressed together with EGFR at high levels, it also inhibits the downregulation of EGFR. Properties of the ligand bound to EGFR can also have a strong influence on receptor degradation. EGFR is efficiently degraded upon EGF stimulation but not upon incubation

with TGF-α. Like EGF, H_2O_2 induces EGFR phosphorylation but to a lower extent than EGF. However, H_2O_2 stimulation of EGFR does not lead to receptor downregulation. This is likely due to the fact that EGFR is not phosphorylated on key tyrosine sites important for receptor downregulation when exposed to H_2O_2. Thus, H_2O_2 seems to induce tumor formation by activating EGFR and uncoupling the activated receptor from normal downregulation, thereby leading to continuous downstream signaling and hence cell proliferation (Zandi et al. 2007).

As mentioned before, the crosstalk of EGFR pathway with other signaling systems that are also dysregulated in cancer is another mechanism of tumorigenesis. In HCC, the crosstalk of the EGFR pathway with the insulin-like growth factor-2 (IGF-2)/IGF-1 receptor (IGF-1R) has been described. IGF-2 exerts its mitogenic effect on HCC cells through AR-mediated transactivation of the EGFR, while its pro-survival activity was mediated through the PI3K pathway (Desbois-Mouthon et al. 2006). COX-2 system states extensive crosstalks with the EGFR axis (Wu 2006). Interestinlgy, c-Met associates with EGFR in tumor cells, and this association facilitates the phosphorylation of c-Met in the absence of hepatocyte growth factor. This cross-talk between c-Met and EGFR may have significant implications for altered growth control in tumorigenesis (Jo et al. 2000). Finally, as mentioned in different parts of this manuscript, a cross-talk between TGF-β and EGF might switch the TGF-β role from tumor suppressor to tumor promoter. In this sense, EGF protects hepatocytes from TGF-β-mediated cell death (Fabregat et al. 1996), a process that occurs upstream from the mitochondria-mediated events (Fabregat et al. 2000). Recent results have indicated that EGF impairs the induction by TGF-β of the NADPH oxidase system (NOX4) responsible for ROS production and cell death (Carmona-Cuenca et al. 2006). These facts are particularly relevant when we take in consideration that TGF-β may transactivate the EGFR pathway (Murillo et al. 2005; Murillo et al. 2007) and the EMT induced by TGF-β is coincident with upregulation of EGFR ligands (Del Castillo et al. 2006).

IV. GROUP BACKGROUND

Over the last years our group has been exploring the different pathways induced by TGF-β in fetal and adult rat hepatocytes. In rat fetal hepatocytes (FH), our group first described that low concentrations of TGF-β (0.5 ng/ml) induced growth inhibition; however, higher concentrations (2 to 5 ng/ml) induced apoptosis (Sanchez et al. 1995; Sanchez et al. 1996). TGF-β-mediated apoptosis was coincident with an increase in the reactive oxygen species (ROS) intracellular levels (beginning at 1 hour of treatment and reaching its maximum after 3-6 hours), which produced a decrease in intracellular glutathione (GSH) levels, all these events being dependent on protein synthesis (Sanchez et al. 1997). ROS increase preceded the down-regulation of Bcl-xL expression, cytochrome c release from the mitochondria to the cytosol, and loss of mitochondrial transmembrane potential (Δψm) (Herrera et al. 2001a; Herrera et al. 2001b). TGF-β-induced caspase-3 activity was not responsible for the rapid decrease of Bcl-xL mRNA transcripts and the initial cytochrome c release observed, but its activation created an amplification through Bcl-xL and c-IAP1 caspase-3-mediated proteolysis (Herrera et al. 2002), mitochondria transmembrane disruption and massive release of cytochrome c. However, when caspases were inactivated by a pan caspase inhibitor (ZVAD), TGF-β-induced cell death was delayed but not completely blocked (Herrera et al. 2001b).

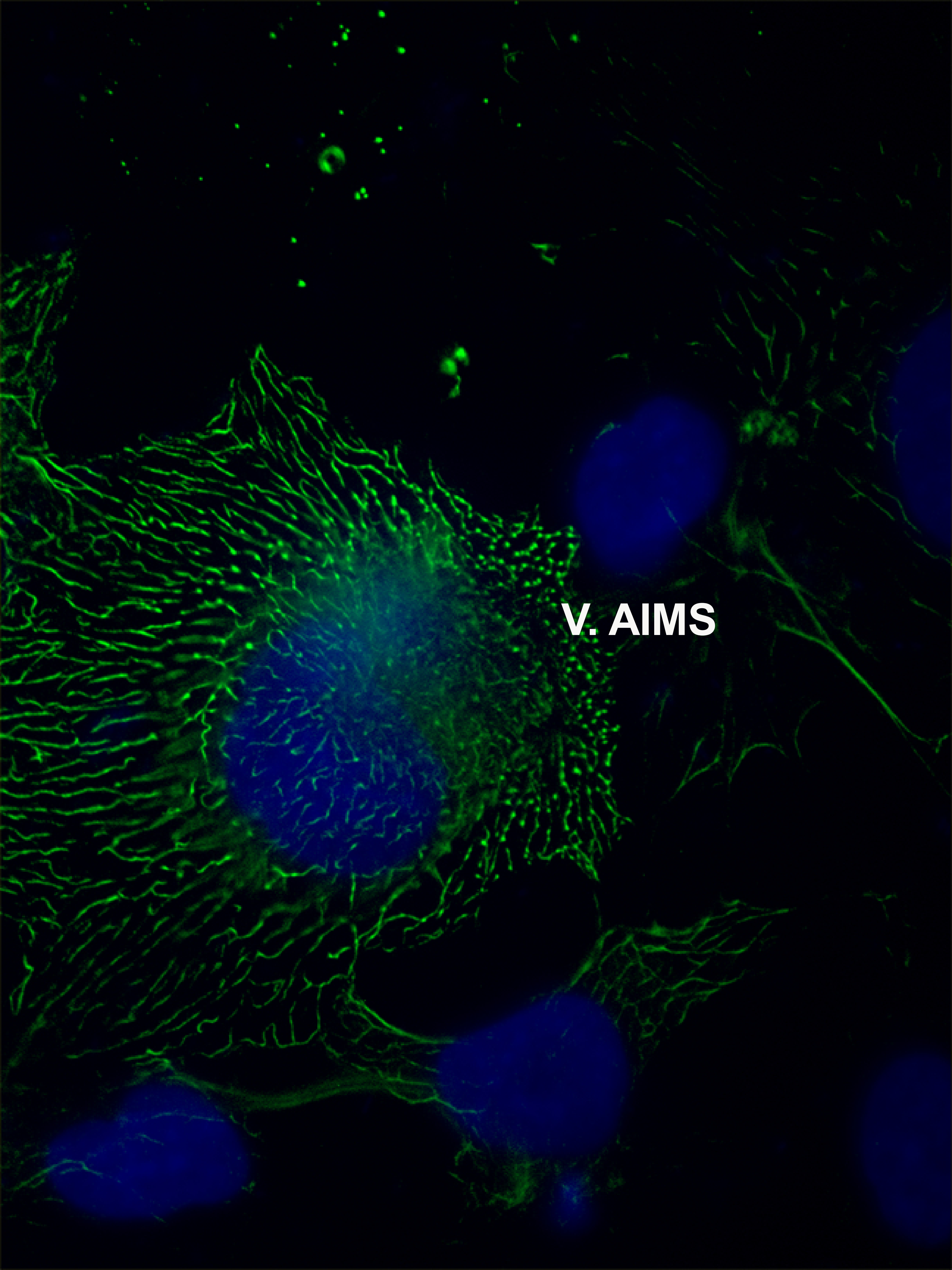
Inhibition of ROS production through the use of antioxidants such as the combination of pyrrolidine carbodithioic acid (PDTC) + ascorbic acid (ASC) blocked all the above-mentioned effects induced by TGF-β (Herrera et al. 2001a). In order to find out the source of ROS, different inhibitors of ROS producing systems were used. Diphenyleneiodonium (DPI), a well-known inhibitor of NADPH oxidase and other flavoproteins, was the only able to completely block TGF-β-induced ROS increase and the subsequent cell death. This result lead us to study whether TGF-β might activate a NADPH oxidase-like system, and indeed such was the case after 1 to 3 hours of treatment. Moreover, TGF-β induced a decrease in the mRNA levels and activity of two of the main antioxidant proteins, Catalase and SOD2 (Herrera et al. 2004). Finally, we discovered that TGF-β up-regulated the expression of Nox4, a homologue of the phagocytic NADPH oxidase Gp91-phox, in rat fetal hepatocytes (Carmona-Cuenca et al. 2006). TGF-β-induced apoptosis was counteracted by pre-incubating rat fetal hepatocytes with Epidermal Growth Factor (EGF) (Fabregat et al. 1996) which, through the activation of the PI3-Kinase pathway, blocked cytochrome c release from the mitochondria to cytosol and loss of Δψm (Fabregat et al. 2000). PI3K mediated EGF protective effects by impairing TGF-β-induced Nox4 expression (Carmona-Cuenca et al. 2006).

However, only 40-50% of the fetal rat hepatocytes died in response to TGF-β, the rest were able to survive to its cytotoxic effects and acquired a fibroblastic-like phenotype (Sanchez et al. 1999). A series of studies were done to understand how some cells survived to TGF-β-induced apoptosis and changed their phenotype in response to this cytokine. It was interesting to find that TGF-β induced both pro- and anti-apoptotic signals (Valdes et al. 2004), the last ones through the activation of the EGF receptor (EGFR) pathway and its downstream survival effectors: c-SRC, PI3K and AKT (Valdes et al. 2004; Murillo et al. 2005). Indeed, EGFR inhibition, with its specific inhibitor the tyrphostine AG1478, enhanced TGF-β-induced apoptosis. To activate the EGFR pathway, TGF-β rapidly increased the activity of the metalloprotease TACE/ADAM17, responsible for EGF ligands shedding (Murillo et al. 2005), and also increased the levels of TGF-α and HB-EGF, through induction of their gene expression, a process that required NF-κB activation and was dependent on NAPDH oxidase activity (Del Castillo et al. 2006; Murillo et al. 2007). Cells that survived to TGF-β responded to this cytokine in terms of migration and invasion. After 24 hours of treatment the surviving cells showed a fibroblastic-like morphology, the actin cytoskeleton was reorganized into stress fibers and there was replacement of the intermediate filaments from cytokeratin 18 (CK-18) to Vimentin. This change in

morphology was coincident with decreased expression of hepatic markers such as albumin and alpha-fetoprotein, and lower expression and DNA–binding activity of liver-enriched transcription factors (HNF1α and HNF4) (Fabregat et al. 1996; Sanchez et al. 1999). TGF-β induced a process of epithelial-mesenchymal transition (EMT), coincident with down-regulation of E-cadherin expression and up-regulation of Snail, a key player in EMT (Valdes et al. 2002). Although primary fetal rat hepatocytes in culture cannot be maintained for longer than few days and trypsinization induces their cell death, the fibroblastic-like population after TGF-β treatment could be expanded in culture (TGF-β treated fetal hepatocytes: TβT-FH). TβT-FH cells had higher expression of Bcl-xL and basal activation of AKT, and were resistant to TGF-β-induced cell death. These cells showed mesenchymal characteristics such as: 1) expression of Vimentin, alpha-smooth muscle actin (SMA) and Fibronectin; 2) down-regulation of E-cadherin and up-regulation of its transcriptional repressor, Snail; 3) higher migratory capacity (Valdes et al. 2002). Moreover, TβT-FH had suffered a de-differentiation process, which conferred them characteristics of liver stem cells. Indeed, 1) they expressed low levels of AFP, Albumin, Connexin 26 or transthyretin, proteins that are characteristic of the hepatocyte lineage; 2) expressed stem cell markers, such as OV6, Thy1 and c-Kit; 3) could be re-differentiated towards a bile duct lineage when cultured with DMSO + EGF, or towards an hepatocytic lineage when cultured with a differentiation media (del Castillo et al. 2008). This TGF-β-induced transdifferentiation process might have implications both in liver fibrosis and hepatocarcinogenesis.

Finally, preliminary results at the beginning of this doctoral thesis had revealed that the dual response to TGF-β observed in fetal rat hepatocytes in primary culture was exclusive of this period of the liver development, since TGF-β induced only apoptosis in rat adult hepatocytes. The inability of TGF-β to induce survival signals appeared to be due to the very low expression of both AKT and TACE observed in adult hepatocytes. Furthermore, TGF-β was unable to induce an EMT process in adult rat hepatocytes. In view of these results, we considered of a great relevance to analyze which might be the response to TGF-β in liver tumor cells.

Our goal was to analyze whether liver carcinoma cells respond to TGF-β-induced cell death and in the case that they had acquired resistance, to study the molecular mechanisms. We also wanted to know whether TGF-β induces survival signals and an EMT process in liver tumor cells, and the potential relevance in liver tumor progression. Although we wanted to start the study with rat liver hepatoma cells, due to our experience in this cell model, we considered very important to analyze the situation also in human liver tumor cells, because it is well known that TGF-β levels are elevated in hepatocellular carcinoma (HCC) and different evidences have suggested that TGF-β response is altered in HCC cells.


V. AIMS

General Objective:

TO ANALYZE THE INTRACELLULAR SIGNALS INDUCED BY TGF-β IN LIVER TUMOR CELLS TO UNDERSTAND ITS ROLE IN HEPATOCARCINOGENESIS.

Specific Objectives:

1. Analysis of the TGF-β response in liver tumor cells in terms of apoptosis. Role of Nox4. Putative role of EGFR in TGF-β-induced survival signals.

2. Analysis of the intracellular survival signals, independent of the EGFR pathway, that may counteract TGF-β-induced apoptosis in human hepatocarcinoma cells.

3. Effects of TGF-β on liver cell phenotype and differentiation state.

VI. MATERIAL AND METHODS

1. Cell Culture

1.1. Liver tumor cell lines: FaO, Hep3B, HepG2, PLC/PRF/5, SK-Hep1

All cell lines were obtained from the European Collection of Cell Cultures (ECACC). FaO rat hepatoma cells were maintained in F12 Coon's Modified medium. HepG2 and Hep3B cells were maintained in MEM medium; PLC/PRF/5 cells were maintained in DMEM medium, and SK-Hep1 cells were cultured with MEM supplemented with 1 mM Sodium Pyruvate. The molecular characteristics of the human HCC cell lines used in this study is presented in Table I. All media were supplemented with 10 % fetal bovine serum and maintained in a humidified atmosphere of 37°C, 5% CO2. All cell lines need to be split at sub-confluent cultures (70-80 %) by trypsinization. For experiments, cells at 70% confluence were serum-starved during 8 to 12 hours before treatments. After this time it is considered that cells lose the proliferative stimuli obtained from fetal bovine serum components.

Table I. Molecular characteristics of the human HCC cell lines used in this study

Cell line	Tumor Type	Morphology	P53 status	HBV	Other Characteristics
Hep3B	Human Negroid hepatocyte carcinoma	Epithelial	Deleted	Integrated	Deficient in functional pRB; mutations within hFas gene
HepG2	Human Caucasian hepatoblastoma	Epithelial	Intact	-------------	Mutation in NRAS
PLC/PRF/5	Human liver hepatoma cells	Epithelial	Mutation pR249S	Integrated	
SK-Hep1	Human liver adenocarcinoma	Endothelial	Intact	-------------	Mutation in CDKN2A (p.R249S) and in BRAF (pV600E)

1.2. Human Fetal Hepatocytes

Human fetal hepatocyte primary cultures were performed in the Department of Pathology, University of Washington, Seattle (USA) during a stay in Nelson Fausto PhD. Laboratory.

Human fetal livers were obtained from Central Laboratory for Human Embryology at the University of Washington in accordance with a protocol approved by the institutional review board of the university. Tissue obtained from legally aborted first- and second-trimester fetuses between 87 and 127 days of gestation was minced in Seglen's collagenase buffer (Table II) containing 3 mg/mL collagenase I using sterilized scissors. The cell suspension obtained is digested during 20 minutes at 37°C under gentle agitation; after 10 minutes of incubation, cells are pipetted up and down to help their disaggregation. Cells were collected by centrifugation at 700 rpm for 5 minutes, resuspended in 0.5 mM EGTA buffer (Table III), and successively passed through 23-gauge needles to yield a single cell suspension. Cells were collected by centrifugation at 700 rpm for 5 minutes and resuspended in their Seeding medium (Table IV). Typical yields were $1x10^7$ cells, cells were seeded in collagen-

coated tissue culture plates as follows: 1.5 million cells per 100 mm dish, 0.5 million cells per 60 mm dish, 0.25 million cells per 35 mm dish, and 0.05 million cells per well of 24-well plates.

Table II. Seglen's Buffer, pH 7.4

NaCl	3.90 g/l
KCl	0.5 g/l
$CaCl_2 \cdot 2H2O$	0.7 g/l
Hepes	24 g/l
NaOH 8 N	8.25 ml/l

Table III. EGTA Perfussion Buffer pH 7.4

EGTA	19 mg/l
NaCl	8 g/l
KCl	350 mg/l
KH_2PO_4	16 mg/l
$MgSO_4 \cdot 7H2O$	16 mg/l
Hepes	2.4 g/l

Table IV. Seeding Media for HFH

Material	Final Concentration	
William's E Medium		Gibco-Invitrogen 12551-032-500 ml
Nicotinamide	10 mM	Sigma N-0636
Ascorbic Acid	0.2 mM	Sigma A 8960-5g
Sodium Bicarbonate	17 mM	Gibco-Invitrogen 25080-094-100 ml
Hepes Buffer 1M	20 mM	Gibco-Invitrogen 15630-080-100 ml
Glucose Stock (50g/200ml)	14 mM	Sigma G5767-500g
Sodium Pyruvate	550 mg/L	Gibco-Invitrogen 11360-070 100 ml
L-Glutamine	2 mM	Gibco-Invitrogen 25030-081-100 ml
ITS + Premix (Insulin, Transferrin, Acid Selenous)	10 ml/L	BD Biosciences 354352-20 ml
Penicillin-Streptomycin	120 μg/ml-100 μg/ml	Gibco-Invitrogen 15140-122-100 ml
Dexamethasone	10^{-4} mM	Sigma D-1756 100 mg
EGF (mouse or human)	20 ng/ml	BD Biosciences 354052 hEGF
Fetal Bovine Serum	5 %	Hyclone Corporation

After 16 hours of seeding the cells, the media was changed to the same media but without fetal bovine serum to which 20 ng/ml EGF were added. The media was changed every 48 hours during 10 days. After this period of time, cells were ready to be used for experiments, their media was change to a seeding media without FBS, EGF, ITS+ premix, nicotinamide, ascorbic acid, sodium piruvate and dexamethasone for 16 hours, at which point they can be treated with TGF-β and different inhibitors.

1.3. Treatments used in cell culture

Different pharmacological inhibitors were incubated 30 minutes before adding 2 ng/ml of TGF-β:

PD98059 (MEK inhibitor)	50 μM
U0126 (MEK inhibitor)	10 μM
PD0325901 (MEK inhibitor)	2 μM
AG1478 (EGFR inhibitor)	20 μM
Gefitinib/Iressa/ZD1839 (EGFR inhibitor)	2.5 μM
TAPI-1 (TACE inhibitor)	100 μM
LY290042 (PI3K inhibitor)	20 μM
PD169316 (p38 inhibitor)	800 nM
SP600125 (JNK inhibitor)	40 μM
Glutathione-ethyl-ester (GEE, membrane-permeable form of GSH)	2 mM
Diphenyleneiodonium (DPI, a flavoprotein inhibitor)	10 μM
Pepstatin A (inhibitor of aspartic proteinases such as pepsin, cathepsins D and E)	100 μM
Ca-074 (N-(L-3-trans-propylcarbamoyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline, Cathepsin B inhibitor)	20 μM
Calpain III inhibitor	100 μM
3-Methyl-Adenine (3-MA, Autophagy inhibitor)	10 mM

1.4. Cell viability analysis

1.4.1. Crystal Violet Staining

This method allows quantifying the amount of cells that survive after a toxic process, and it consists on cell staining with a colorant, crystal violet. This method is only useful when working with adherent cells that detached after undergoing a toxic process (Drysdale et al. 1983). After the cells are incubated, in 12 or 24-well plates, with the different stimuli or inhibitors, the cell media is removed, and cells are washed twice with PBS. Then, a solution of Crystal violet at 0.2 % (w/v) in 2% ethanol is added during 30 minutes. Following this, the staining solution is removed, and the wells are washed several times with PBS or distilled water until the excess staining that has not been incorporated into the cells is eliminated. The plate is air-dried, and the stained cells are lysed in 10% SDS. By spectrophotometric analysis, the absorbance is measured at 595 nm. The results are then calculated as the percentage of viable cells relative to control cells (cells incubated in the absence of growth factors or inhibitors) at the indicated time at each experiment.

1.4.2. Multitox-Fluor Multiplex Cytotoxicity Assay (Promega, Madison, USA)

Cells are seeded in 96-well plate. Once the time of treatment has elapsed, Multitox-Fluor Multiplex Cytotoxicity Assay (Promega, Madison, USA) reagents are added as indicated by manufacturer's protocol. Dead cells fluorescence and life cells fluorescence are measured in a Microplate Fluorescence Reader Fluostar Optima. Viability was calculated as a ratio of live/dead cells fluorescence, and expressed as percentage of untreated cells (cells incubated in the absence of growth factors or inhibitors). This method was preferentially used in cells that remain attached to the dish even although they have died (as an example, HepG2 cells).

2. Analysis of cell proliferation

2.1. DNA synthesis assay

To study DNA synthesis, the method of ^{3}H-Thymidine incorporation was used. Cells can convert [^{3}H]-Thymidine into [^{3}H]-Thymine, which can then be incorporated into DNA as it is synthesized. This procedure allows the quantification of only newly synthesized DNA. Cells are seeded in 12 or 24-well plate and serum depleted 8 to 12 hours before the different treatments. Four hours after addition of the extracellular factors a mix of radioactive and cold Thymidine (0.5 µCi/ml, 1 µM cold Thy) is added. After 48 hours of treatment, wells are washed with PBS, and the cellular products precipitated by adding to the well cold 10 % TCA, during 20 minutes at 4°C. Next, the wells are washed twice with cold 70 % ethanol and let dry. Finally, a solution containing 0.1 N NaOH, 2% CO_3Na_2 and 0.5% SDS is added to the well, which is incubated during 20 minutes at 37°C. 100 µl from each well is mixed with 5 ml of scintillation fluid and radioactivity counted for [^{3}H] in a liquid scintillation counter. Results are shown as percentage of each treatment relative to untreated cells.

2.2. Analysis of the percentage of cells in each phase of the cell cycle.

Once cells have been treated with different factors and/or inhibitors, they are tryspsinized and both media and cells are collected in a 15 ml tube, which is then centrifuged at 2500 rpm for 5 min. The pellet contains those cells that were attached to the tissue culture dish and those that were dead and floating in the media. The pellet is resuspended in 200 µl of PBS 1X and is added drop by drop into 500 µl of cold 100% Ethanol. At this point, samples can be stored at -20°C. To collect the fixed cells, samples are centrifuged at 2500 rpm during 5 minutes at 4°C. The pellet is air-dried and resuspended in 250 µl PBS that contains 0.1 mg/ml of RNAse. After incubation during 30 minutes at 37°C, propidium iodide is added at a final concentration of 0.05 %. This suspension is analyzed in a flow cytometer FACSan from Becton-Dickinson at the Serveis Cientifico-Tecnics, Universitat de Barcelona. Cell cycle analysis was carried out using the software ModFit LT™ (Verity Software House): DNA content: 2C: G0/G1 phases; 4C: G2/M phases; >2C and <4C: S phase.

3. Analysis of cell death

3.1. Analysis of caspase-3 activity

Once cells have been treated with the different extracellular factors, media is preserved and cells are scrapped. Then, cells and media are collected together in a 15 ml tube, which is then centrifuged at 2500 rpm for 5 min. Indeed, this pellet contains those cells that were attached to the tissue culture dish and those that were dead and floating in the media. The pellet is resuspended in 30 to 100 µl lysis buffer, Table V. The solution is then transferred to an Eppendorf tube, which is incubated for 20 minutes on ice and vortexed every 10 minutes. After this time has elapsed, the Eppendorfs are centrifuged at 13000 rpm during 10 minutes at 4°C. The supernatant is stored at -20 or -80°C until it is used.

To determine Caspase-3 Activity, first we determine the protein concentration by Bradford's method. Then a mix containing 20 µg of protein (in a final volume of 25 µl), 125 µl of Buffer Reaction 2X (Table VI) and 2 µl of fluorogenic substrate for caspase-3, Ac-DEVD-AMC (BD Pharmigen) is prepared. This substrate, once it is cleaved by caspase-3, releases the AMC fragment that is fluorogenic and can be quantified by spectrofluorimetry. After 2 hours of incubation at 37°C, fluorescence is measured using an exciting wavelength of 360 nm and an emission wavelength of 440 nm.

Table V. Caspase-3 Lysis Buffer	
Tris-HCl pH 8	5 mM
EDTA	20 mM
Triton-X-100	0.5 %

Table VI. Caspase-3 Reaction Buffer 2X	
Hepes pH 7.5	40 mM
Glycerol	20 %
DTT	4 mM

A unit of caspase-3 activity is the amount of active enzyme necessary to produce an increase in 1 fluorescence unit in the spectrofluorimeter. Results are usually represented as Arb unit/h/µg protein. In some figures, data are expressed as fold induction, or percentage of increase, versus control.

3.2. Analysis of the percentage of cells with a DNA content lower than 2C

The intracellular DNA content is analyzed by flow cytometry, as described in the section 2.2. Cells with a DNA content lower than 2C (hypodiploid cells) are cells in a process of apoptosis that have suffered DNA fragmentation.

3.3. Analysis of the percentage of cells containing active Bax or Bak

Cells are plated on gelatin-coated glass coverslips. The monolayer is washed with PBS, cells are fixed with 4% paraformaldehyde in PBS for 30 min at RT, and permeabilized with 0.1% Triton X-100-1% BSA (2 min). Primary antibodies, anti-Bax antibody 6A7 clone and anti-Bak G317-2 clone (BD Pharmingen) (1:50) are diluted in 1% BSA and incubated for 16 h at RT. After several washes with PBS, the samples are incubated with fluorescent-conjugated secondary antibodies (1:200 for Alexa

Fluor 488-conjugated anti-mouse) for 1 h at RT and embedded in Vectahield with DAPI (Vector Laboratories, Burlingame, CA). Cells are visualized in an Olympus BX-60 microscope with the appropriate filters. Blue signal represents the nuclear DNA staining with DAPI. Representative images are taken with a Spot 4.3 digital camera and software and edited in Adobe Photoshop. Positive cells are counted and results are shown as percentage of positive cells relative to cell number.

3.4. Analysis of mitochondrial and lysosomal transmembrane potential

The fluorescent probes LysoTracker Red DND-99 and MitoTracker Red CMXRos (Invitrogen) were used to analyze lysosome membrane permeabilization and mitochondrial transmembrane potential, respectively. Cells are trypsinized and centrifuged at 2500 rpm during 5 minutes at 4°C. The fluorescent probes are loaded into the cells by incubation in HBSS without red phenol at a final concentration of 50 nM for 30 min at 37°C, and transferred in duplicate into a 96-well plate. Fluorescence is measured in a Microplate Fluorescence Reader Fluostar Optima using an exciting wavelength of 510 nm and an emission wavelength of 590 nm. Results are calculated as fluorescence units per µg protein and then expressed as percentage of control.

4. Measurement of intracellular redox state

In order to measure the intracellular content of ROS, a fluorimetric method was used. Each treatment was analyzed in triplicate. After treatment of cells, cultured in 12-well plates, they are washed with PBS and incubated with 2.5 µM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) in HBSS without red phenol during 30 minutes at 37°C in the dark. This compound is incorporated into the cells, and converted into 2’, 7’-diclorodihidrofluorescein ($DCFH_2$) by intracellular sterases, that in turn is converted into 2’, 7’-diclorofluorescein (DCF) when oxidized by hydrogen peroxide. Then cells are lysed with 250 µl of the lysis buffer described in Table VII during 10 minutes at 4°C, and transferred in duplicate into a 96-well plate, each with 100 µl. Intracellular GSH content was also analyzed fluorimetrically. After the time of treatment, cells are collected by trypsinization, pelleted and resuspended in 250 µl of 2 mM Monochlorobimane in HBSS. This suspension is plated in duplicate in a 96-well plate, each with 100 µl, and incubated during 1 hour at 37°C. The resting 50 µl are lysed with the buffer described in Table VII and used for protein quantification. Fluorescence is measured in a Microplate Fluorescence Reader Fluostar Optima using an exciting wavelength of 485 nm and an emission wavelength of 520 nm, in the case of ROS measurement, or an exciting wavelength of 355 nm and an emission wavelength of 440 nm for analysis of GSH content. Cell auto-fluorescence (produced by cells incubated with HBSS without fluorimetrical probe) is substracted. Results are calculated as fluorescence units per µg protein and then expressed as percentage of control.

Table VII. Lysis Buffer	
Hepes 1 M pH 7.5	25 mM
NaCl	60 mM
$MgCl_2$	1.5 mM
EDTA	0.2 mM
Triton-X-100	1 %

5. Analysis of gene expression

5.1. RT-PCR

RNeasy Mini Kit (Qiagen, Valencia, CA) is used for total RNA isolation. Reverse transcription (RT) was carried out with random primers using 0.2 to 1 μg of total RNA from each sample for complementary DNA synthesis using High Capacity RNA to cDNA Master Mix Kit (Applied Biosystems) following manufacturer instructions.

5.2. Semi-quantitative PCR

Semi-quantitative Polymerase Chain Reactions (PCR) are performed using 5 μl of 1:10 diluted cDNA, using a final concentration of 1 μM of rat (rn, rattus novergicus) or human (hs, homo sapiens) specific primers (Table VIII), dNTPS at 100 μM, 1.5 mM $MgCl_2$ and 1 unit of Ecotaq (Ecogen). PCR reactions are carried out following the conditions shown in Table IX.

Table VIII. Primer sequences used in semi-quantitative PCR

Gene	Forward Primer	Reverse Primer	Tm (ºC)	Nº Cycles
rn-Tgfa	5'TGGTGCAGGAAGAGAAGC 3'	5'TGACAGCAGTGGATCAGC 3'	59	35
rn-Hbegf	5'CGGTGGTGCTGAAGCTCTTTC 3'	5'TGGTAACCAGGGAGGCAGTG 3'	59	35
rn-Alb	5'CTGCCGATCTGCCCTCAATAGC 3'	5'GTGCCCACTCTTCCCAGGTTTCT 3'	58	30
rn-Snai1	5'GCAGCTGGCCAGGCTCTCGGTGGC 3'	5'GTAGCTGGGTCAGCGAGGGCCTCC 3'	65	35
rn-Cdh1	5'CGTGATGAAGGTCTCAGCC 3'	5'ATGGGGGCTTCATTCAC 3'	64	35
rn-Tgfb	5'CCTGCTGCTTTCTCCCTCAACC 3'	5'CTGGCACTGCTTCCCGAATGTC 3'	58	35
rn-Hnf1a	5'CGGACTGATTGAAGAGCCCAC 3'	5'CTGGTTGAGACCTGGAGACGT 3'	63	30
rn-Hnf1b	5'TCAGTCAACAGAACCAGGGCC 3'	5'GCCGGGGAGACTTGTTGTAAA 3'	63	30
rn-Hnf3b	5'GCACCTGAGTCCGAGTCTGAG 3'	5'GAGCTGAACCTGAGAAGCCTG 3'	63	30
rnHnf4a	5'AGTACATCCCGGCCTTCTGTG 3'	5'GACCCTCCAAGCAGCATCTCC 3'	63	30
rn-kit	5'AGCAAGAGTTAACGATTCCGGAG 3'	5'CCAGAAAGGTGTAAGTGCCTCCT 3'	58	40
rn/hs-18S	5'GCGAAAGCATTTGCCAAGAA 3'	5'CATCACAGACCTGTTATTGC 3'	53	25
hs-AR	5'TGGTGCTGTCGCTCTTGATACTCG 3'	5'CCCACACCGTTCACCGAAATATTC 3'	55	30
hs-HBEGF	5'AGACAGACAGATGACAGCACCACAGC 3'	5'AGACAGACAGATGACAGCACCACAGC 3'	55	30
hs-TGFA	5'GTTCGCTCTGGGTATTGTGTTGGC 3'	5'TTTCGGACCTGGCAGCAGTGTATC 3'	55	30
hs-TACE	5'CATTATCAAACCCTTTCCTGCG 3'	5'TCCTTCTCAAACCCATCCTCG 3'	55	30
hs-ALB	5'TGCTTGAATCTGCTGATGACAGGG 3'	5'TGTCTTGGCAAGTCTCAGCAGCAG 3'	55	30
hs-NOX4	5'AACACCCTGTTGGATGACTGGAAAC 3'	5'GGACCTCCTATAAACAGTCTTGAATTC AGT 3'	55	35
hs-SMAD2	5'GGAGCAGAATACCGAAGGCA 3'	5'CTTGAGCAACGCACTGAAGG 3'	60	30
hs-SMAD3	5'AGAAGACGGGGCAGCTGGAC 3'	5'GACATCGGATTCGGGGATAG 3'	58	30
hs-SMAD4	5'CATCGACAGAGACATACAG 3'	5'CAACAGTAACAATAGGGCAG 3'	56	30
hs-SMAD6	5'CAAGCCACTGGATCTGTCCGA 3'	5'TTGCTGAGCAGGATGCCGAAG 3'	59	30
hs-SMAD7	5'ATGCTGTGCCTTCCTCCGCT 3'	5'CGTCCACGGCTGCTGCATAA 3'	59	30
hs-TGFBR1	5'CGTGCTGACATCTATGCAAT 3'	5'AGCTGCTCCATTGGCATAC 3'	58	30
hs-TGFBR2	5'GCACGTTCAGAAGTCGGTT 3'	5'AGATATGGCAACTCCCAGTGGT 3'	56	30
hs-p22-PHOX	5'TCCTGCATCTCCTGCTCTC 3'	5'CACAGCCGCCAGTAGGTAG 3'	62	30
hs-SNAI1	5'CGAAAGGCCTTCAACTGCAAAT 3'	5'ACTGGTACTTCTTGACATCTG 3'	50	35
hs-CDH1	5'TCCCATCAGCTGCCCAGAAA 3'	5'TGACTCCTGTGTTCCTGTTA 3'	55	30
hs-CDH2	5'TGGAACGCAGTGTACAGAATCAGT 3'	5'TTGACTGAGGCGGGTGCTGAATT 3'	55	30
hs-VIM	5'TGAGGCTGCCAACCGGAACAATGAC 3'	5'ATTCTGAATCTCATCGTGCAGGCG 3'	60	30

Table IX. PCR Conditions			
Phase	**Temperature (ºC)**	**Time**	**Nº Cycles**
Initial Denaturalization	95	5 min	1
Denaturalization	94	30 s	25-40
Hybridization	55-65	30 s	25-40
Elongation	72	30-45 s	25-40
Final Elongation	72	7 min	1

The obtained PCR products are analyzed in 1.5 % agarose gels in Ethidium Bromide, in the following buffer: 40 mM Tris-HCl, 0.1 % glacial acetic acid, 1 mM EDTA.

5.3. Real Time PCR

RNA is obtained as explained in the above section. PCR reactions are done in duplicate in a 96-well plate, in a final volume of 25 µl. The reaction is prepared using 25 µl of Taqman ® Universal Master Mix, 2.5 µl of predesigned Taqman ® primer (Table X), and 2 µg of cDNA plus RNAse free water up to 50 µl per duplicate. Gene expression is determined in an ABIPrism 7300 System following Manufacturer's protocol at the IDIBELL Serveis Tècnics Centrals. The levels of mRNA for each gene are normalized with the housekeeping gene Gapdh

Table X. predesigned Taqman ® primer	
Primer	**Catalogue number**
rn-Nox4	rn00585380_m1
rn-Gapdh	rn99999916_s1
hs-GAPDH	hs99999905_m1
hs-NOX4	hs00276431_m1
hs-EGFR	hs01076092_m1
hs-CD90/THY1	hs00174916_m1
hs-CD117/c-KIT	hs00174029_m1
hs-EpCAM	hs00901885_m1
hs-ALB	hs00910225_m1
hs-AFP	hs00173490_m1

When gene expression was analyzed in human fetal liver, it was performed by SYBR green using rotor-gene 3000 (Corbett Research, Sydney, Australia) at the Department of Pathology, University of Washington, Seattle, USA. It was performed using 40 cycles, at a hypridization temperature of 58 to 62ºC. At the Table XI, the primer sequences are specified.

Table XI. Primer sequences used in SYBR real time PCR			
Gene	**Forward Primer**	**Reverse Primer**	**Tm (ºC)**
hs-SNAI1	5’CGAAAGGCCTTCAACTGCAAAT 3’	5’ACTGGTACTTCTTGACATCTG 3’	50
hs-CDH1	5’TCCCATCAGCTGCCCAGAAA 3’	5’TGACTCCTGTGTTCCTGTTA 3’	55
hs-CDH2	5’TGGAACGCAGTGTACAGAATCAGT 3’	5’TTGACTGAGGCGGGTGCTGAATT 3’	55
hs-VIM	5’TGAGGCTGCCAACCGGAACAATGAC 3’	5’ATTCTGAATCTCATCGTGCAGGCG 3’	60
hs-GAPDH	5’ATTGCCCTCAACGACCACTTT 3’	5’TTGATGGTACATGACAAGGTGCGG 3’	55
hs-NOX4	5’CTCAGCGGAATCAATCAGCTGTG 3’	5’AGAGGAACACGACAATCAGCCTTAG 3’	55
hs-AFP	5’TGCAGCCAAAGTGAAGAGGGAAGA 3’	5’CATAGCGAGCAGCCCAAAGAAGAA 3’	55
hs-ALB	5’TGCTTGAATGTGCTGATGACAGGG 3’	5’AAGGCAAGTCAGCAGGCATCTCATC 3’	55
hs-HNF4A	5’CCAAGTACATCCCAGCTTTC 3’	5’TTGGCATCTGGGTCAAAG 3’	55

5.4. RT-MLPA

RNA was analyzed by reverse transcriptase multiplex ligation–dependent probe amplification (RT-MLPA) using SALSA MLPA KIT R011 Apoptosis mRNA from MRC-Holland (Amsterdam, The Netherlands) for the simultaneous detection of 40 messenger RNA molecules. In brief, RNA samples (200 ng total RNA) are first reverse transcribed using a gene-specific probe mix. The resulting cDNA is annealed overnight at 60°C to the MLPA probe mix. Annealed oligonucleotides are ligated by adding Ligase-65 (MRC-Holland, Amsterdam) and incubated at 54°C for 15 minutes. Ligation products are amplified by PCR (33 cycles, 30 seconds at 95°C; 30 seconds at 60°C, and 1 minute at 72°C) with one unlabeled and one FAM labeled primer. The final amplified PCR fragments are separated by capillary electrophoresis on a 48-capillary ABI-Prism 3730 Genetic Analyzer (Applied Biosystems/Hitachi, Foster City, CA). Peak area and height are measured using GeneScan analysis software (Applied Biosystems). The levels of mRNA for each gene are expressed as a normalized ratio of the peak area relative to the peak area of a control gene, β2-microglobulin. The probe set contains probes for mRNAs of 30 apoptosis-related genes (for some genes there are probes for different splice variants of a certain mRNA) and 5 house-keeping genes used for normalization.

5.5. Knock-down assays

For transient siRNA transfection, Hep3B at 70 % of confluence were transfected using TransIT-siQuest (Mirus, Madison, USA) at a 1:300 dilution in complete medium, according to the manufacturer’s recommendation, with a final siRNA concentration of 50 nM. Experiments were carried out as explained in the scheme Fig. 1A.

Table XII. siRNA sequences		
gene	**sequence**	**concentration**
NOX4	5’GCCUCUACAUAUGCAAUAA 3’	25 nM(HepG2) 50 nM (Hep3B)
EGFR	5’GCGAUAAGUCGUGUCUUAC 3’	50 nM
ERK1	5’UUGCGCACGUGGUCAUAGG 3’	200 nM
ERK2	5’GUACAGGACCUCAUGGAAA 3’	200 nM
Unspecific	5’GUAAGACACGACUUAUCGC 3’	25 to 200 nM

For transient siRNA transfection, HepG2, PLC/PRF/5 and SK-Hep1 cells at 70% confluence were transfected using TransIT-siQuest (Mirus, Madison, USA) at a 1:300 dilution in complete medium, according to the manufacturer's recommendation, with a final siRNA concentration of 25 to 200 nM during 8 hours. After 16 hours of incubation in complete medium, cells were trypsinized and plated for experiments. Oligos were obtained from Sigma-Genosys (Suffolk, UK). The oligo sequences are shown in Table XII. The unsilencing siRNA used was selected from previous works (Sancho et al. 2006). Specific oligos with maximal knock-down efficiency were selected among three different sequences for each gene. Experiments were carried out as explained in the scheme Fig. 1B.

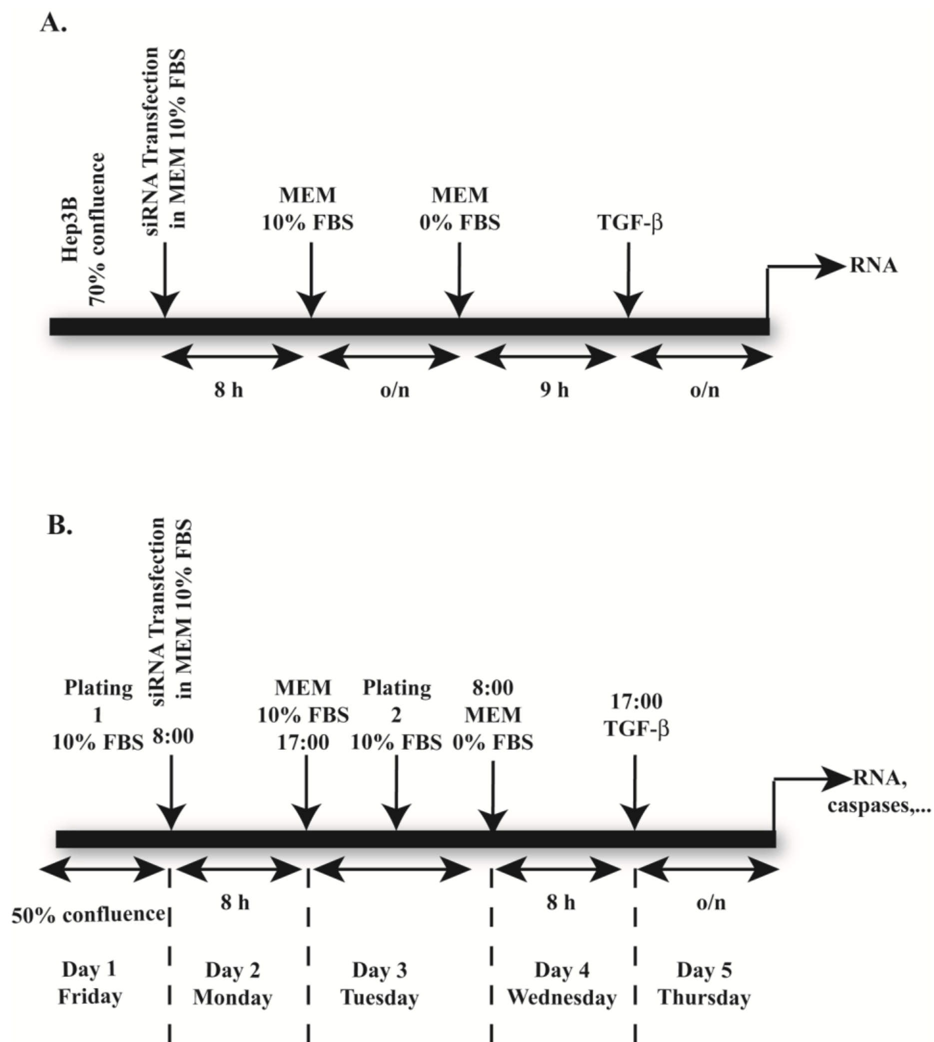


Figure 1. SiRNA Transfection Protocol. A. Hep3B cells. B. HepG2, PLC/PRF/5 and SK-Hep1 cell lines

6. Protein expression analysis

Once cells have been treated with different factors, the tissue culture dishes are placed on ice. The media is collected into tubes, 2 ml of cold PBS are added to each dish, and cells are scrapped and collected to the tube. To make sure that most of the cells are collected, other 2 ml of cold PBS are added to the dish and collected into the tube. Cells are then pelleted at 2500 rpm during 5 minutes at 4°C. The pellet is then washed with 5 ml of PBS, and centrifuged at 2500 rpm during 5 minutes at 4°C. The pellet is resuspended with lysis buffer (Table XIII) and transfered to an eppendorf tube, the lysis is performed during 1 hour with rotation at 4°C. Then, the tubes are centrifuged at 13000 rpm during 10 minutes at 4°C, and the supernatants are collected and saved at -20 °C or -80°C until they are processed.

Table XIII. Lysis buffer	
Sodium deoxicolate	1 %
Tris-HCl pH 7.4	10 mM
SDS	0.1 %
Triton-X-100	1 %
NaCl	150 mM
EDTA	2 mM
PMSF	1 mM
Leupeptin	5 μg/ml
Na3VO4	0.1 mM
DTT	0.5 mM
β-glicerolphosphate	20 mM

6.1. Protein quantification by Bradford's method.

Protein quantification is done following the spectrophotometric method described by M. Bradford (Bradford 1976). For each measurement a standard curve of protein concentration is prepared with BSA in a range from 0 to 2 μg/ml. The reaction is prepared mixing 200 μl of distilled water, 2 μl of protein extract and 50 μl of Bradford reaction. The absorbance is measured at 595 nm.

6.2. Protein quantification by BCA commercial kit.

If SDS is used in the lysis buffer, then the Bradford method cannot be used. In these cases, we use the commercial kit BCA. For each measurement a standard curve of protein concentration is prepared with BSA in a range from 0 to 2 μg/ml. The reaction is prepared by mixing Solution A and B in a ratio of 50:1 and 200 μl of this mix are added to 10 μl of 1:10 diluted sample into a 96-well plate. After 30 minutes of incubation at 37°C, the absorbance is measured at the spectrophotometer at 595 nm.

6.3. Protein immunodetection by Western blot

Protein separation by their molecular weight is done by denaturalizing polyacrylamide gels. The protein samples are prepared by mixing 30 to 100 μg of protein with Laemmli buffer, and are denaturalized by heating them at 95 °C. Once the samples are boiled, they are spinned and saved at 4 °C. Acrylamide gels consist of two different parts: the stacking and the separating gel, the last one being prepared at different concentrations of acrylamide depending on the size of the proteins to be studied. For instance, when phospho-kinases were analyzed, a gel of 10 or 12 % acrylamide was used, when BCL-2 family members were analyzed a 15 % acrylamide gel was used. Once the gel is ready it is assembled into the gel holder and immerse into the tank, which is filled with a electrophoresis buffer (25 mM Tris-HCl; 0.1% SDS; 0.2 M glycine; pH 8,3). Then the samples are carefully loaded to the gel together with a molecular weight standard in order to know the molecular weight of the proteins studied. Then they are submitted to electrophoresis following the method described by M. Sambrook (Sambrook et al. 1989), at a constant voltage.

Once finished the electrophoresis, the proteins are transferred to a PVDF membrane through the passage of electrical current using a semi-dry equipment. The PVDF membrane is "activated" by its immersion into methanol for one minute following the manufacturer's instructions. Then the PDVF membrane and the Wattman paper are soaked in Transfer Buffer (Table XIV) for 5 minutes, and the equipment is assembled as follows from bottom to top: 3 Wattman papers-PVDF membrane-Acrylamide gel-3 Wattman papers. An electrical current of 0.3 mA is used during 0.5-1 hour. After this time, the membrane is stained into a solution of 0.5 % red Ponceau in 1% acetic acid to confirm whether the transfer has worked and the proteins have uniformly been transferred into the membrane. Then, it is washed several times in TTBS (Table XV)

Table XIV. Transfer Buffer	
Tris-HCl pH 8.3	48 nM
Glycine	39 nM
SDS	0.04 %
Methanol	20 %

Table XV. TTBS	
Tris-HCl pH 7.5	10 nM
NaCl	100 nM
Tween-20	0.05 %

The membrane is then incubated in 5% non fat dry milk in TTBS for 1 hour at room temperature (RT). After this time, it is incubated with the primary antibody in 0.5 % milk-TTBS at the dilution indicated at Table XVI for 16 hours at 4°C. Then the membrane is washed 3 times during 5 minutes with TTBS, and incubated with the secondary antibody at a dilution of 1:5000 in 0.5 % milk-TTBS during 1 hour at RT. Again the membrane is washed several times in TTBS. To visualize the antibody hybridized specifically to the protein of study, the membrane is incubated with a chemiluminescent solution, ECL, from Amersham Biosciences.

The secondary antibodies used were obtained from GE-Healthcare: Anti-Mouse (NA931V), and anti-Rabbit (NA934V), they are conjugated with peroxidase.

Table XVI. Antibodies used for Western Blot			
Primary Ab	**Dilution**	**Secondary Ab**	**Purchased from**
Phospho-EGFR	1:1000	Rabbit	Cell Signaling 2234
EGFR	1:1000	Rabbit	Cell Signaling 2232
Phospho-AKT	1:1000	Rabbit	Cell Signaling 9271
AKT	1:1000	Rabbit	Cell Signaling 9272
Phospho-SRC	1:1000	Rabbit	Cell Signaling 2101
Phospho-JNK	1:1000	Rabbit	Cell Signaling 9251
Phospho-ERK1/2	1:1000	Rabbit	Cell Signaling 9101
ERK1/2	1:1000	Rabbit	Cell Signaling 9102
Phospho-SMAD2	1:1000	Rabbit	Cell Signaling 3101
β-ACTIN	1:3000	Mouse	Sigma AC-14 A5441
BIM	1:1000	Rabbit	BD Pharmigen 559685
BMF	1:1000	Rabbit	Abcam 9655
BCL-XL	1:1000	Rabbit	Santa Cruz 634
MCL1	1:1000	Rabbit	Santa Cruz 819
NOX4	1:1000	Rabbit	Sigma-Genosys against a peptide corresponding to the C-terminal loop region (aminoacids 499-511).
P53	1:200	Mouse	Neomarkers MS-18
FOXA2/HNF3β	1:500	Goat	Santa Cruz 9187
CX26	1:1000	Rabbit	Zymed 71-0500
VIM	1:1000	Mouse	Sigma V6630
CK18	1:1000	Mouse	Progen #61028
HO-1	1:1000	Rabbit	Sigma H4535
SOD2	1:1000	Rabbit	Upstate 06-984
CATALASE	1:1000	Mouse	Sigma C0979
γ-GCS	1:1000	Rabbit	Abcam 17926

The hybridized antibody can be removed by stripping the membrane during 30 minutes at 50°C with the Stripping Solution described in Table XVII.

Table XVII. Stripping Solution	
Tris HCl pH 6.8	62.5 nM
SDS	0.05 %
β-mercaptoethanol	100 nM

6.4. Immunoprecipitation

Once cells have been treated with different factors, cells are collected as explained in section 6. In order to enrich the sample in the amount of Phospho-tyrosine proteins, 300-500 µg of proteins are diluted in lysis buffer (Table IV) to a final concentration of 1µg/µl (up to 500 µl); then the samples are incubated in Eppendorf tubes with an anti-phosphotyrosine Antibody (Clone 4G10 from Millipore, 1:100) for 16 hours with rotation at 4°C. Protein G-Sepharose beads coupled to a rabbit anti-mouse bridge antibody are then added to the lysates for additional 3-hours incubation at 4°C. Previously, G-Sepharose beads (25 µl/sample) are washed 3 times in lysis buffer, to finally be resuspended in 12.5 µl lysis buffer/sample. After the incubation of the sample with the Protein G-Sepharose beads, the immunoprecipitates are washed four times with 1 ml lysis buffer. The pellet containing the protein G-Sepharose coupled to phospho-tyrosine is resuspended in Laemmli sample buffer, and boiled. Protein concentration of the supernatant is quantified and 50 µg are loaded to the acrylamide gel as the "input" (non-tyrosine phosphorylated protein). Denatured proteins are separated by a 10 % SDS-polyacrylamide gel electrophoresis (PAGE). Proteins are then transferred to a PVDF membrane for western blotting, following the explanations in section 6.3.

7. Immunocytochemistry

7.1. Fluorescence microscopy studies

Cells are plated on gelatin-coated glass coverslips. For F-actin staining, after treatment, cells are washed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at RT and incubated with rhodamine-conjugated phalloidin (1:500) diluted in 0.1% BSA for 1 h. To detect albumin, E-cadherin and cytokeratin-18, cells are fixed with cold methanol for 2 min. Primary antibodies, see Table XVIII (1:50) are diluted in 1% BSA and incubated for 2 h at RT. For EGFR staining, cells are fixed with 4% paraformaldehyde for 12 min at RT, permeabilized in PBS containing 0.1% triton X-100-0.1% BSA for 3 min, blocked with 1%BSA and 10%goat serum in PBS for 1 h and then incubated with anti-EGFR antibody (1:100) diluted in 1% BSA, overnight at 4°C. After several washes with PBS, the samples are incubated with fluorescent-conjugated secondary antibodies (anti-mouse Alexa 488, anti-rabbit Alexa 488 or anti-rabbit Cy3-conjugated), at 1:200 for 1 h at RT and embedded in Vectahield with DAPI (Vector Laboratories, Burlingame, CA). Cells are visualized in an Olympus BX-60 microscope with the appropriate filters. Blue signal represents the nuclear DNA staining with DAPI. Representative images were taken with a Spot 4.3 digital camera and software and edited in Adobe Photoshop. When indicated, a Leica TCS-SL confocal microscope, with the x63 Leitz Plan-Apo objective, was used.

Table XVIII. Primary Antibodies for ICC

Antibody	Purchased from
Phalloidin	Sigma P1951
E-Cadherin	BD Trnasduction 610181
CK18	Progen #61028
Albumin	Nordic Immunological Laboratories
Vimentin	Sigma V6630

7.2. Visible microscopy studies

Cells are plated on collagen-coated glass coverslips and, after treatments, rinsed with PBS to remove residual medium. Then, cells are fixed with Methanol:Acetone (50:50) at -20ºC for 5 minutes. The coverslips can be dried and stored at -20ºC. Before use, they need to be rehydrated in PBS for a minimum of 10 minutes. Then the endogenous peroxidase is blocked in 0.3 % peroxide solution (4 ml of Methanol + 5.9 ml PBS + 0.1 ml 30% Hydrogen peroxide) for 10 to 20 minutes at RT. After this time of incubation, preparations are washed a minimum of 3 times in PBS for 5 minutes each time. Then the coverslips are incubated in blocking solution, 2%FBS-PBS, for 10 minutes. The blocking solution is removed and the primary antibody (See Table XIX) is added at 1:50 dilution in 2% FBS-PBS during 1 to 2 hours of incubation at RT. Then, preparations are washed a minimum of 3 times in PBS for 5 minutes each time. Detection of the primary antibody is carried out using the appropriate biotinylated antibody (Vectastain, Burlingame, CA) at a 1:100 dilution in 2%FBS-PBS for an hour at RT. Again, the preparations are washed 3 times in PBS for 5 minutes, and incubated in ABC solution for half an hour. Finally, the antibodies are detected with the peroxidase DAB kit (Ventana, Tucson, AZ). The preparations are counterstained with 3X Gill's Hematoxillin for 5 minutes. Preparations were analyzed using digital photography (Olympus DP11: Olympus America, Melville, NY) and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Table XIX. Primary Antibodies for ICC in HFH

Antibody	Purchased from
CK18	ICN Pharmaceuticals
Vimentin	SIGMA
SMA	DAKO M0851
Hepar1	DAKO
A1AT	DAKO A0012
Albumin	ICN Pharmaceuticals
CX26	Zymed 71-0500
CK7	DAKO M7018
CK19	Amersham Pharmacia
EpCAM	R&D CP63
CD90 (THY)	BD 22081A

8. Statistical Analysis

Differences were analyzed by comparing different treatment by using nonparametric analysis (Mann–Whitney or an paired *t* test with Wilcoxon correction). When comparing different treatments in the absence or presence of an inhibitor a two way ANOVA test was used with Bonferroni-posttest. Data are represented as mean ± SEM with the following symbols indicating the level of significance; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Statistical anlysis was performed by using PRISM software (GraphPad, San Diego).

VII. RESULTS

1. Analysis of the TGF-β response in liver tumor cells in terms of apoptosis. Role of NOX4. Putative role of EGFR in TGF-β-induced survival signals.

1.1. TGF-β plays a dual role, both suppressing and promoting apoptosis, in rat hepatoma cells.

Taking into consideration the results obtained by our group in the recent years, we decided to explore TGF-β effects on liver tumor cells. First, we started analyzing the response of FaO rat hepatoma cells to this cytokine in terms of apoptosis and survival.

TGF-β induced apoptosis in FaO rat hepatoma cells (Fig. 1), in agreement with previous reports (Choi et al. 1998). After 36 h of treatment with TGF-β alone, the number of viable cells was reduced to 50 %. When cells where pre-incubated with a selective inhibitor of the EGFR (AG1478), the percentage of viable cells was further reduced to 40 % and to 20 % after 24 and 36 h of treatment, respectively (Fig. 1A). TGF-β induced caspase-3 activity reaching a maximum at 24 h. This activity was significantly accelerated and increased when both TGF-β and AG1478 were present, reaching its maximum at 12 h (Fig.1B). We also observed an increase in the percentage of hypodiploid (apoptotic) cells after 24 h of treatment with TGF-β, which was significantly increased when AG1478 was also added (Fig. 1C).

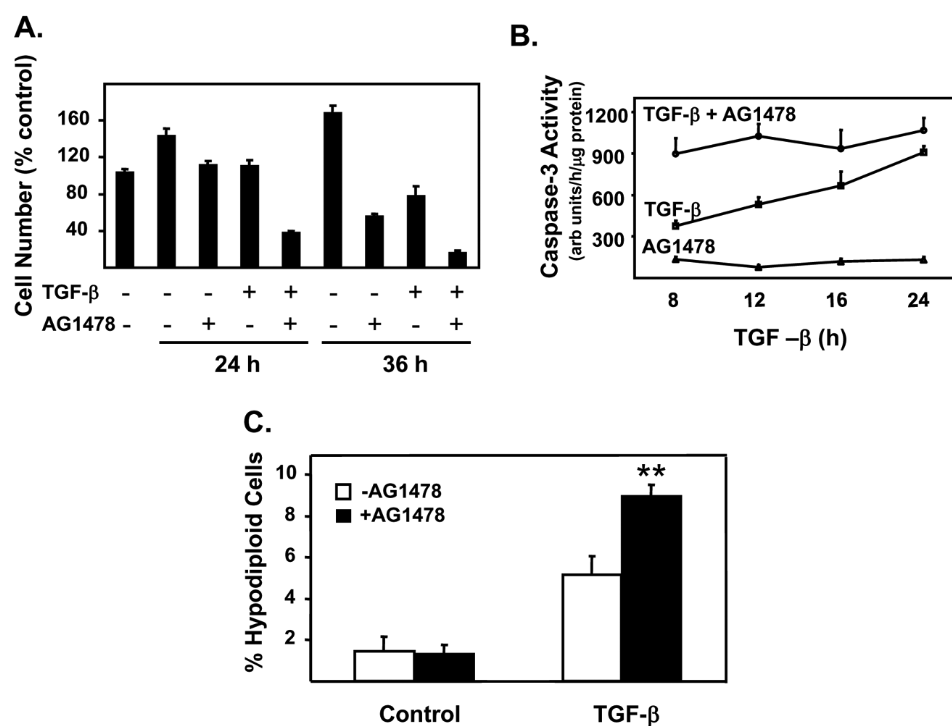


Figure 1. TGF-β-induced apoptosis in FaO rat hepatoma cells is enhanced by inhibition of EGFR. FaO cells were incubated for different times with or without TGF-β and/or AG1478 (20 μM). **A**. Number of viable cells, analysed by crystal violet staining. Results are expressed as percentage of the initial number of cells (left bar) and are mean ± S.E.M. of three independent experiments, in triplicate. **B.** Caspase-3 activity. Results are the mean ± S.E.M. of three different experiments in duplicate. **C.** DNA content, analysed by flow cytometry after 24 h treatment with TGF-β. The percentage of hypodiploid (apoptotic) cells is shown. Data are the mean ± S.E.M. of four different experiments.

TGF-β transiently activated PI3K/AKT and c-SRC Kinase in FaO cells after 1 h of treatment (Fig. 2A). This increased phosphorylation of proteins involved in survival pathways correlated with the phosphorylation of SMAD2, indicating that TGF-βRI was being activated (Fig. 2A).

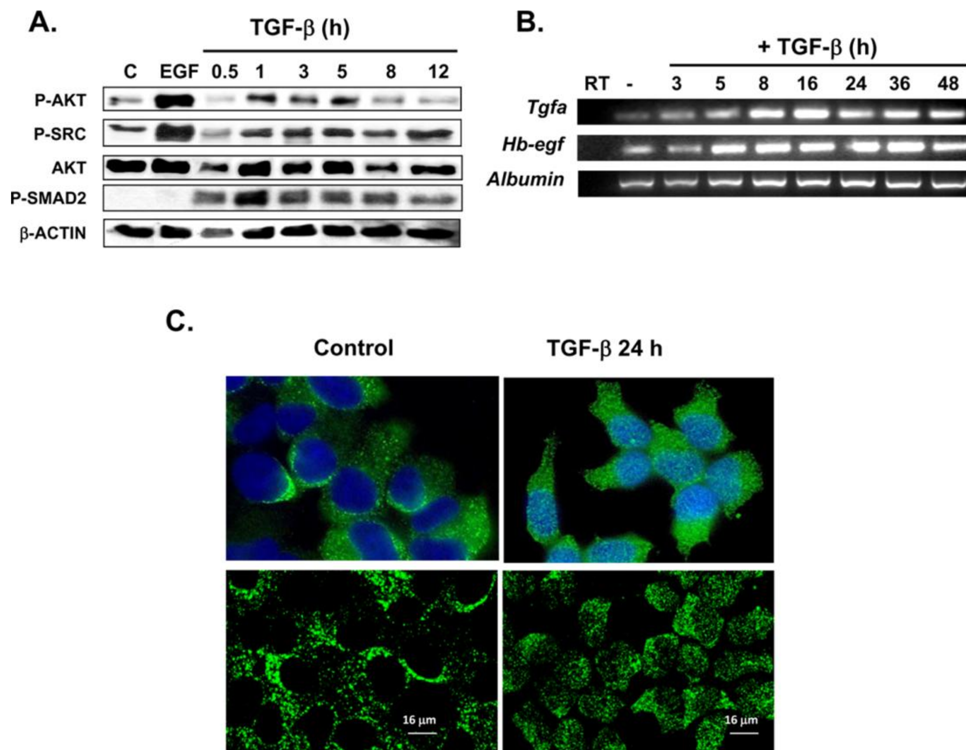


Figure 2. TGF-β activates the EGFR pathway in FaO rat hepatoma cells. A. FaO cells were incubated with TGF-β at different times, as indicated; stimulation with EGF was used as a positive control for AKT and SRC activation. Western Blot analysis, β-ACTIN is used as a loading control. **B**. Levels of *Tgfa* and *Hb-egf* transcripts, analyzed by RT-PCR after different times of treatment with TGF-β. *Albumin* transcripts are shown as control. A representative experiment, of at least five, is shown. **C.** Changes in the intracellular localization of the EGFR (green) after 24 h of TGF-β stimulation in FaO rat hepatoma cells. Upper panels: conventional fluorescence microscopy, combined with DAPI (blue) to observe nuclei. Lower panels: confocal microscopy.

These results led us to study whether the expression of EGFR ligands, such as transforming growth factor-alpha (TGF-α) and heparin-binding epidermal growth factor-like growth factor (HB-EGF) could be induced by TGF-β. RT-PCR analysis showed that the expression of both genes was up-regulated after TGF-β treatment. It is also interesting to mention that under basal conditions the transcription of both *Hb-egf* and *Tgfa* genes is detected, although at a lower level (Fig. 2B), leading us to speculate that an autocrine loop of EGFR ligands might exist in FaO rat hepatoma cells. To demonstrate that the EGFR pathway was being activated by TGF-β, we have analyzed the intracellular localization of the receptor in untreated and treated (24h) cells (Fig. 2C). It is well known that binding of EGFR ligands to the specific receptor in the hepatocyte cell membrane produces internalization of the complex ligand-receptor (Dunn et al. 1986). Furthermore, translocation of this complex to the nucleus has been also suggested (Schausberger et al. 2003). Results presented in Fig. 2C indicated that FaO cells in the absence of TGF-β showed most of the receptor protein localized in the cell periphery, although we could observe protein in the cytosol and/or intracellular vesicles, which suggest that an EGFR turnover exists as a consequence of the autocrine production of EGFR ligands. Interestingly, 24h of treatment with TGF-β produces internalization of the receptor, which is also localized in the nucleus, as evidenced by confocal microscopy (Fig. 2C), corroborating the functional activity of the EGFR ligands whose expression is increased by TGF-β.

To confirm that the survival signals induced by TGF-β were mediated by the EGFR, cells were incubated in the absence or presence of TGF-β with or without AG1478. In all our experiments we detected a basal activation of SRC and AKT that was reversed by AG1478 itself, which confirmed that a basal activation of EGFR exists in these cells. Interestingly, AG1478 also completely avoided the TGF-β-induced transitory increase in phosphorylation of AKT and SRC, whereas it showed no effect on SMAD2 phosphorylation (Fig. 3A). These results together indicate that TGF-β induces both pro- and anti-apoptotic signals in hepatoma cells. Activation of PI3K/AKT and SRC is mediated by the EGFR, probably by amplifying the autocrine loop of EGF receptor ligands.

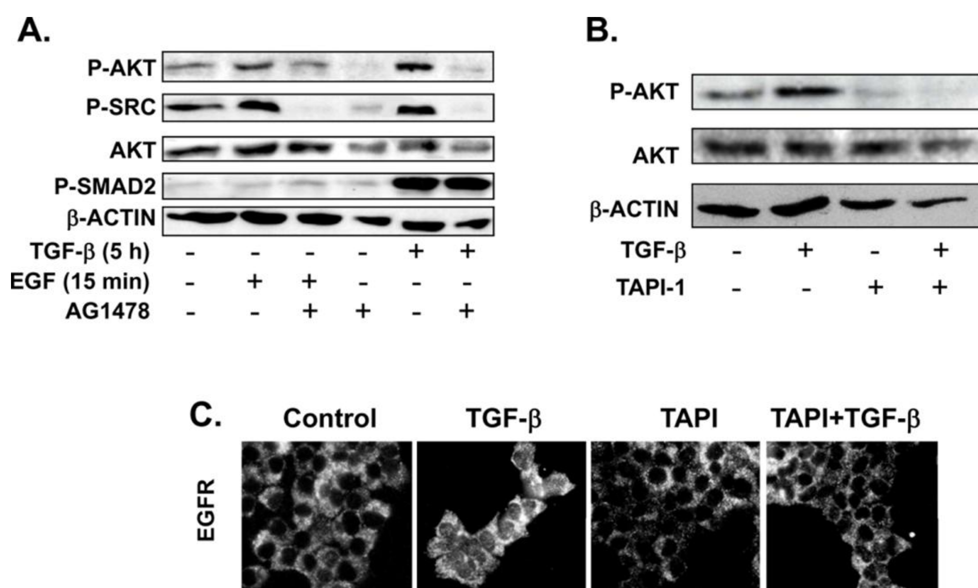


Figure 3. TGF-β-dependent activation of SRC kinase and AKT in FaO rat hepatoma cells: role of the EGF Receptor pathway. A. AG1478 (20 μM) effect on AKT and SRC activation. Western Blot Analysis. Stimulation with EGF (15 min) was used as control for AKT and SRC phosphorylation and efficiency of the AG1478 (20 μM). **B**. Effect of TACE inhibition by its inhibitor TAPI-1 (100 μM) on AKT phosphorylation, Western Blot analysis. In both **A** and **B**, β-ACTIN is used as a loading control. **C.** Changes in the intracellular localization of the EGFR by immunofluorescence after 24 h of TGF-β stimulation in FaO rat hepatoma cells with or without TAPI, conventional fluorescence microscopy. In each case a representative experiment of three is shown.

As mentioned in the Group Background Section, in parallel to these studies, in our group we observed that TGF-β is able to induce cell death in rat adult hepatocytes, but it is unable to induce survival signals (Caja et al. 2007). We had found that adult non-transformed hepatocytes showed a low expression of the metalloprotease TACE, responsible for the shedding of the EGFR ligands. Therefore, we decided to study whether the activation of TACE might be required for TGF-β-induced survival signals in FaO cells. Results indicated that inhibition of TACE/ADAM17 with TAPI-1 blocked the EGFR activation, studied by immunoanalysis of its intracellular distribution (Fig. 3C), and completely inhibited AKT activation by TGF-β (Fig. 3B). These results indicate that TACE/ADAM17 activity is necessary for the survival response to TGF-β in hepatoma cells, which suggests a role of EGFR ligands in this effect.

We have previously described that TGF-β-induced apoptosis is coincident with up-regulation of the NADPH oxidase NOX4 in rat fetal hepatocytes (Carmona-Cuenca et al. 2006), effect that is impaired in the presence of EGF. For this reason, we decided to analyze the levels of NOX4 in cells with TGF-β, combined or not with AG1478. Results indicated that NOX4 was slightly up-regulated by TGF-β, and this up-regulation became highly remarkable when AG1478 was added, both at the mRNA level

(Fig. 4A) and at the protein level (Fig. 4B). These results indicated that in FaO rat hepatoma cells TGF-β-induced apoptosis is also coincident with an increase of NOX4 expression. Autocrine activation of the EGFR pathway, which takes place in these cells even in the absence of TGF-β but increases in its presence, attenuates this effect.

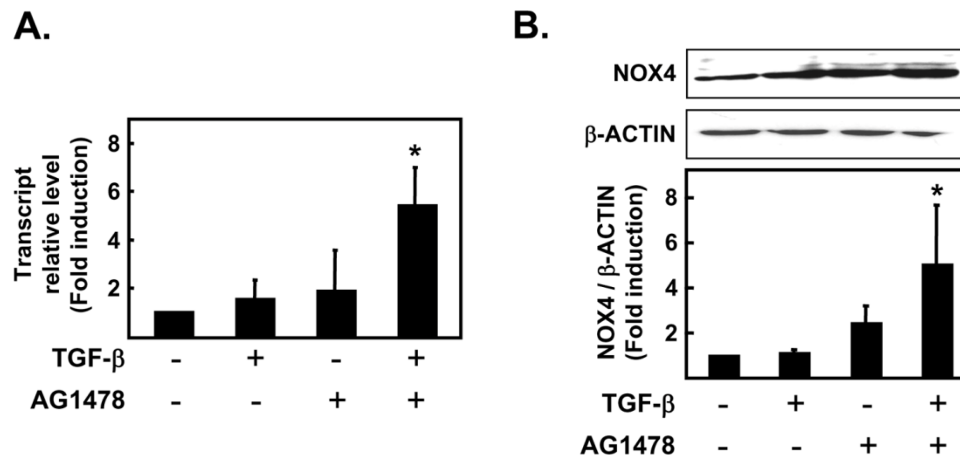


Figure 4. Regulation of Nox4 expression by TGF-β alone or in combination with AG1478. FaO cells were incubated with or without TGF-β and/or AG1478 (20 μM) during 10 hours. **A.** *Nox4* transcript levels determined by Real-Time PCR, results are expressed as mean ± SEM of three independent experiments. **B**. NOX4 protein levels determined by Western Blot, β-ACTIN is used as a loading control. Graphs represent the mean ± SEM of data from densitometric analysis of three different experiments. Student's t test treated cells versus untreated cells: *p<0.05.

1.2. TGF−β induces both pro-apoptotic and anti-apoptotic signals in human fetal hepatocytes and hepatocellular carcinoma cells. Role of the EGFR pathway.

Collectively, the results obtained in rat hepatoma cells drived us to study whether a similar scenario might exist in human hepatocytes. We decided to start by analyzing the response to TGF-β of primary human fetal hepatocytes (HFH) to know whether these cells responded to TGF-β in a dual manner, as fetal rat hepatocytes did (Valdes et al. 2004). For these experiments, we collaborated with Dr. Nelson Fausto at the Department of Pathology, University of Washington. Indeed, all the results along the manuscript performed in primary cultures of human hepatocytes were obtained during a six months-stay in Dr. Fausto's laboratory.

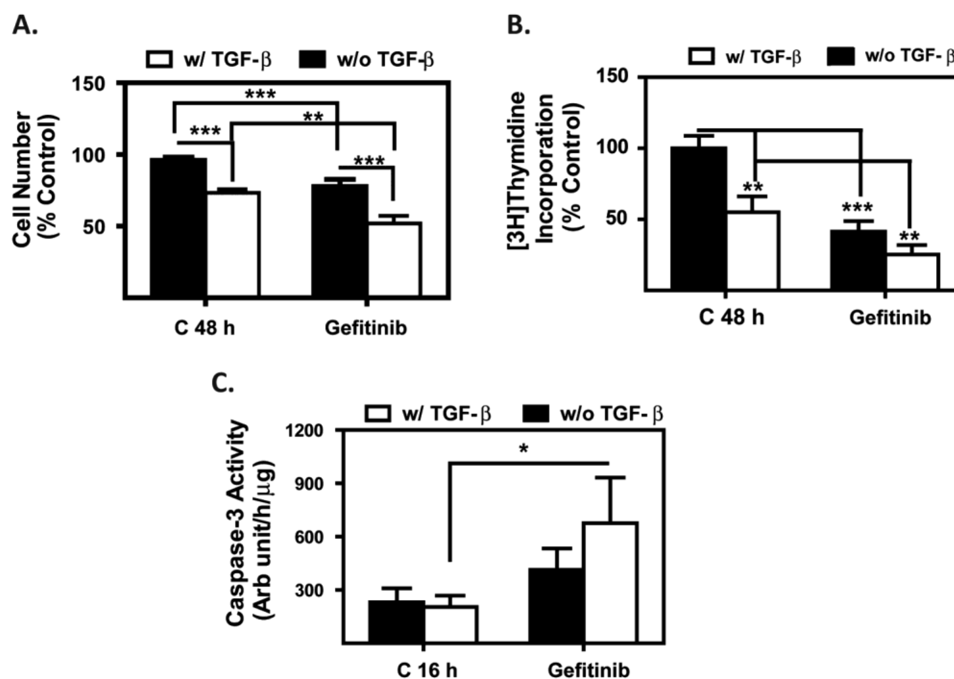


Figure 5. TGF-β-induced cell death in human fetal hepatocytes is enhanced by EGFR inhibition with Gefitinib. Human fetal hepatocytes were incubated with or without TGF-β (1 ng/ml) in the presence or absence of Gefitinib (2.5 µM). **A.** Number of viable cells after 48 hours of treatment, analysed by crystal violet staining. **B.** [3H]-thymidine incorporation into DNA after 48 hours of treatment. **C.** Caspase-3 activity after 16 hours of treatment. Graphs show the mean ± S.E.M. of 3-5 different experiments. Student's t test as indicated in each figure: *p <0.05; **p <0.01; *** p < 0.001.

From a previous work performed by Irene Carmona-Cuenca in Dr. Fausto's lab we knew that HFH responded to TGF-β inducing cell death. Now, we wanted to know whether TGF-β-induced apoptosis would be sensitized by EGFR inhibition. The use of Gefitinib, a selective inhibitor of the EGFR that is currently used in the treatment of locally advanced or metastatic non-small cell lung cancer (NSCLC), not only induced cell growth inhibition as shown by loss of the number of viable cells and a decrease in the percentage of cells that synthesize DNA, but also potentiated TGF-β growth inhibitory effects (Fig. 5A-B). Moreover, Gefitinib potentiated and accelerated TGF-β-induced caspase-3 activation (Fig. 5C), which was coincident with a higher induction of *NOX4* expression (Fig. 6A). We used the RT-MLPA technique to study the TGF-β-induced changes in the expression of BCL-2 and IAP families, the main families involved in the regulation of mitochondrial-mediated apoptosis, and whether or not Gefitinib might have an effect on this. TGF-β treatment induced the expression of the pro-apoptotic *BMF*, and *BIM*, but, simultaneously, it also enhanced the transcript levels of anti-apoptotic genes of the BCL-2 family, particularly *BCL-XL* and caspase inhibitors, such as *XIAP* (Fig. 6B). These results confirmed the dual role played by TGF-β in the control of apoptosis, as we had previously described in rat fetal hepatocytes (Valdes et al. 2004). Interestingly, the presence of Gefitinib attenuated TGF-β-induced up-regulation of the anti-apoptotic *BCL-XL* and *XIAP* (Fig. 6B), but dit not increase the induction of pro-apoptotic genes. It is worthy to note that TGF-β induced a decrease in the expression of the caspase inhibitor *HIAP1*, which was enchanced in the presence of gefitinib.

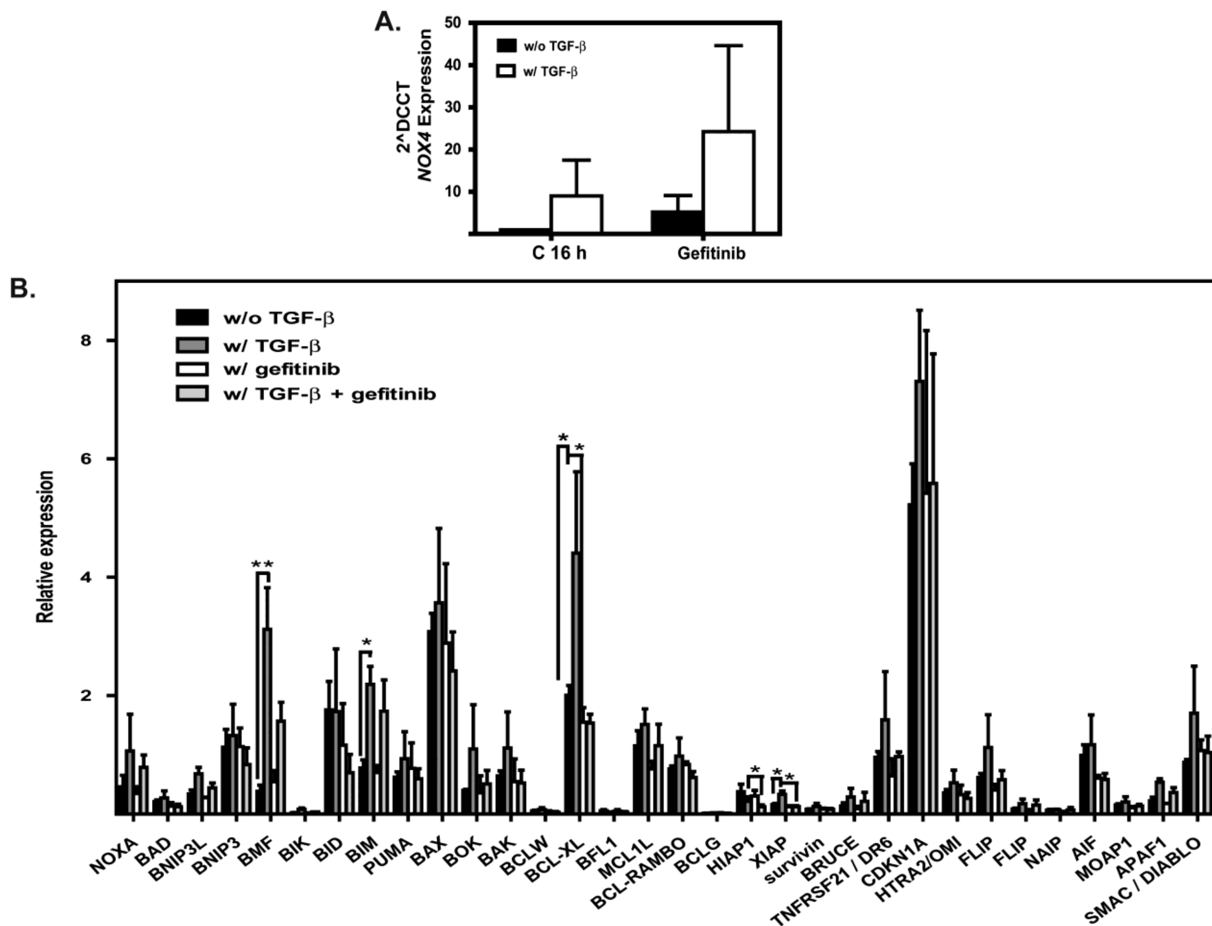


Figure 6. TGF-β and Gefitinib combination regulates the expression of NOX4 and some BCL-2 family members in HFH. Human fetal hepatocytes were incubated with or without TGF-β (1 ng/ml) in the presence or absence of Gefitinib (2.5 μM) during 16 hours. **A.** Analysis of *NOX4* expression by real time PCR. **B.** Transcript levels of apoptotic genes by RT-MLPA expressed as relative expression. Mean ± S.E.M. (n=3). Student's t test untreated cells versus TGF-β-treated cells: $**p < 0.01$; $*** p < 0.001$.

Next, we wondered how human liver tumor cells would respond to TGF-β. First, we analyzed the response of four different human hepatocarcinoma cell lines (Hep3B, HepG2, PLC/PRF/5, cell lines that have an epithelial-like phenotype; and SK-Hep1 cell line which have a mesenchymal-like phenotype) to TGF-β in terms of viability. As can be observed in Fig. 7, Hep3B and PLC/PRF/5 showed autocrine proliferation in the absence of serum. The increase in cell number was significantly attenuated when cells were treated with TGF-β. On the contrary, TGF-β had no effect on the viable cell number of HepG2 and SK-Hep1 cells; indeed SK-Hep1 cells proliferation was enhanced in the presence of TGF-β.

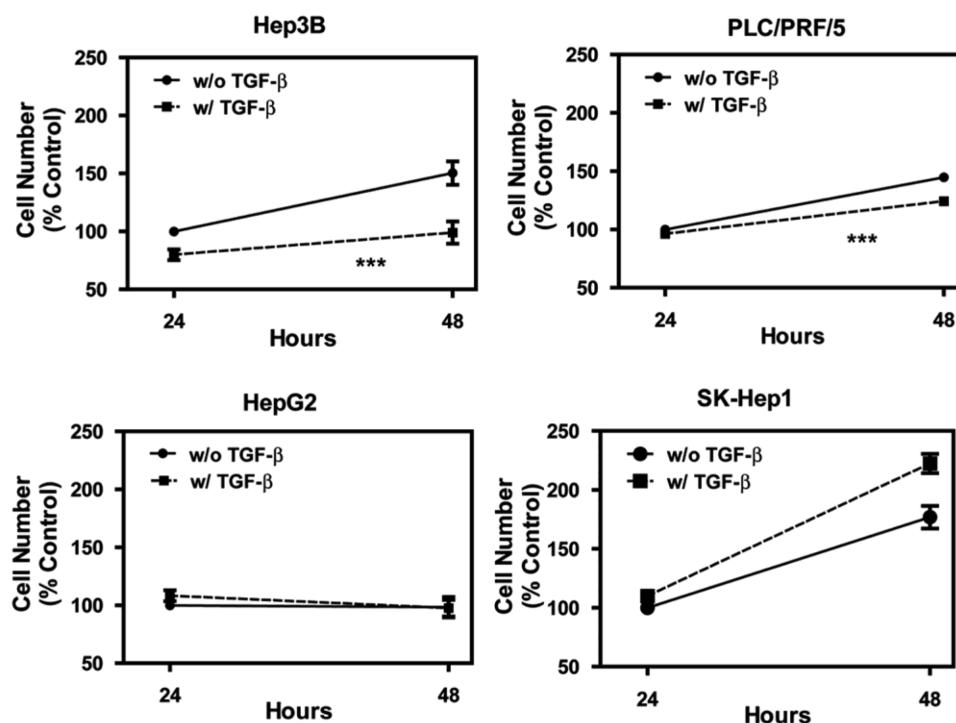


Figure 7. Effects of TGF-β in hepatocellular carcinoma cell lines. Cell viability of Hep3B, PLC/PRF/5, HepG2 and SK-Hep1 cells in the absence or in the presence of 2 ng/ml TGF-β (at the indicated times) was analyzed by Crystal Violet. Results are expressed as percentage of the initial number of cells and are mean $\pm$ S.E.M. of three independent experiments, in triplicate. Student's t test treated cells versus untreated cells: ***$p<0.001$.

We next selected two cell lines, one sensitive and one resistant to TGF-β suppressor effects (Hep3B and HepG2 cell lines, respectively) to analyze the differences in the signaling induced by TGF-β. In Hep3B cells, TGF-β induced the activation of caspase-3 starting after 16 hours of treatment and achieving its peak at 24 hours (Fig. 8A), which preceded the increase in the percentage of hypodiploid cells (Fig. 8B). In contrast, HepG2 cells in the presence of TGF-β did not activate caspase-3 (Fig. 8C), and did not induce DNA fragmentation (Fig. 8D). In Hep3B cells, TGF-β also induced a decrease of the mitochondrial transmembrane potential (Fig. 9A), coincident with up-regulation of the expression of the pro-apoptotic BCL-2 family members BIM and BMF both at the mRNA and protein level, and *BIK* at the mRNA level (Fig. 9B). However, again, a dual role on the expression of apoptosis-related genes is observed, since TGF-β increased the transcript levels of *BCL-XL* and *MCL1*, both anti-apoptotic members of the BCL-2 family (Fig. 9B). In the case of MCL1, a clear increase was also observed at the protein level. However, BCL-XL protein levels did not change, which might be explained by the fact that BCL-XL is substrate of caspases in the TGF-β-induced apoptosis (Herrera et al. 2001b) and up-regulation at the transcription level might be attenuated by the caspase-3-mediated cleavage of the protein.

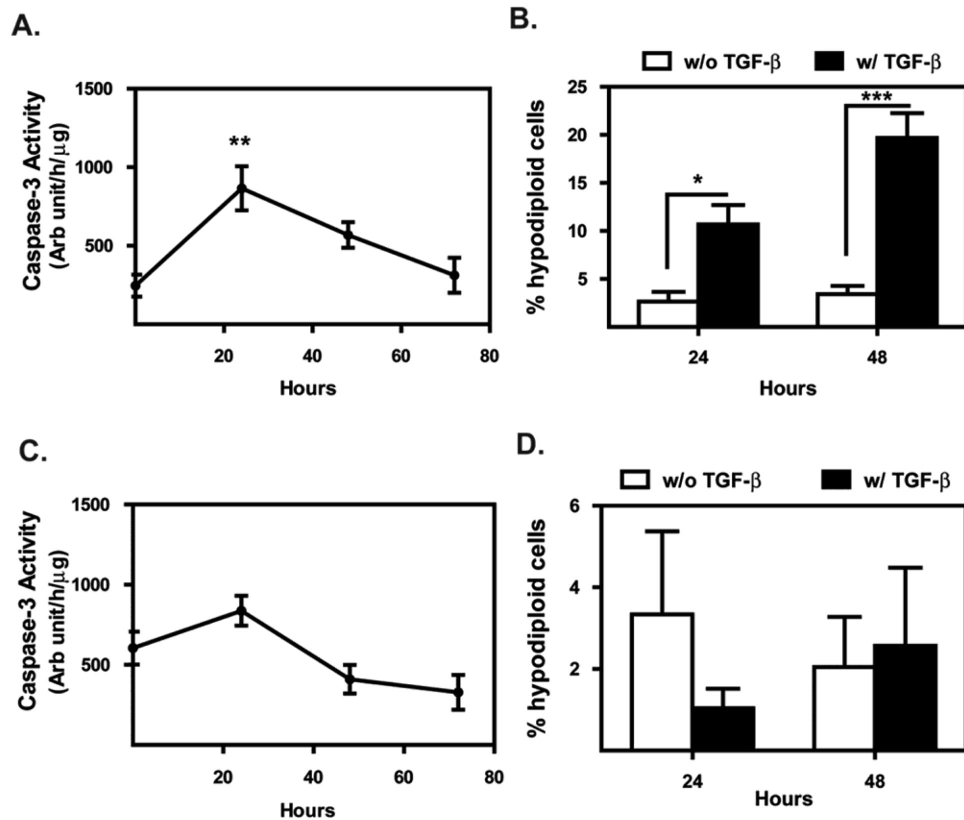


Figure 8. TGF-β-induced apoptosis in Hep3B, but not in HepG2 cells. Hep3B (A, B) and HepG2 (C, D) cells were incubated with TGF-β, 2 ng/ml, at the indicated times. **A, B.** Analysis of Caspase-3 activity. **B, D**. DNA content, analysed by flow cytometry. The percentage of hypodiploid (apoptotic) cells is shown. Graphs show the mean ± S.E.M. of 3-5 different experiments. Student's t test treated cells versus untreated cells: *p <0.05; **p < 0.01; ***p<0.001.

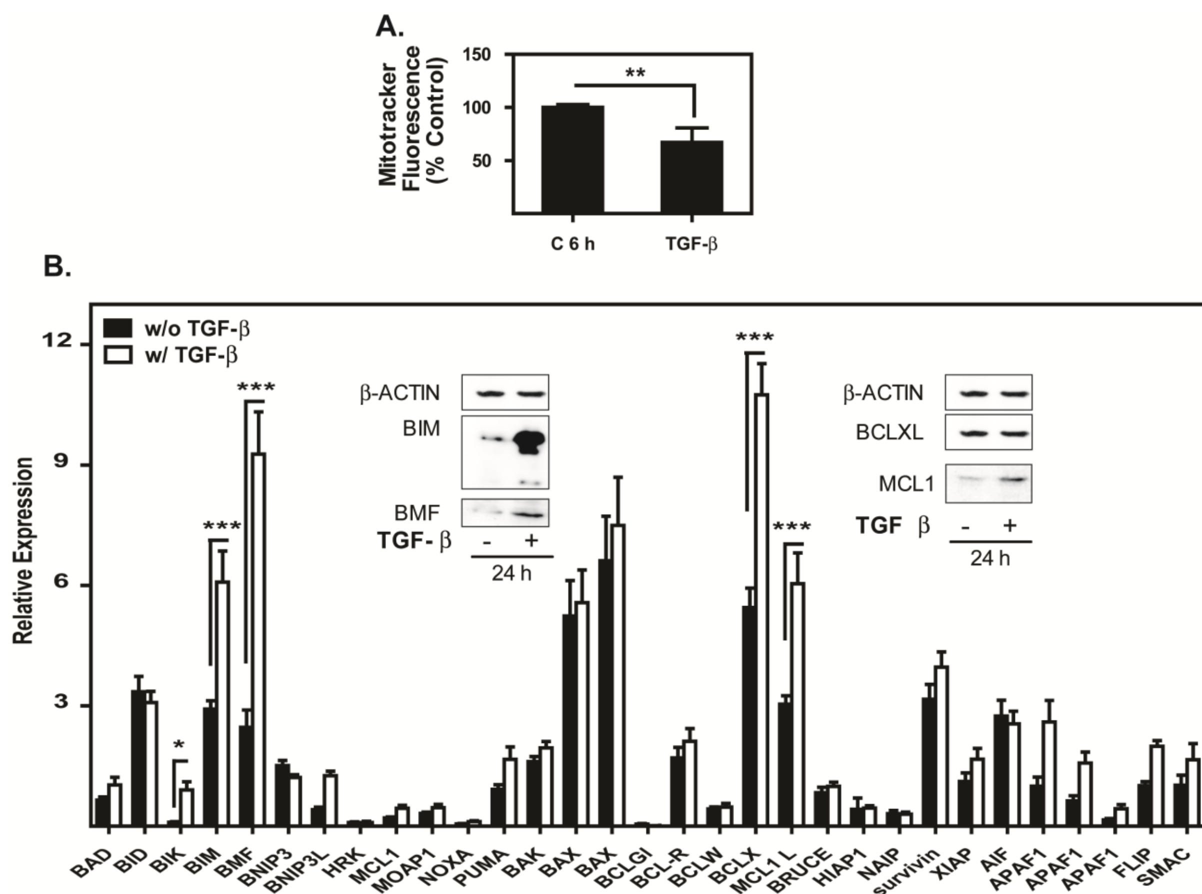


Figure 9. TGF-β decreases mitochondrial transmembrane potential and regulates expression of the BCL-2 family in Hep3B cells. Cells were incubated in the absence or the presence of TGF-β (2 ng/ml). **A.** Analysis of mitochondrial transmembrane potential (mitotracker) after 6 hours of treatment. Mean ± S.E.M. of 3 different experiments. **B.** Transcript levels of apoptotic genes by RT-MLPA after 16 hours of treatment. Mean ± S.E.M. of 4 different experiments, expressed as relative expression. Top: Western Blot analysis (24 h of TGF-β treatment), β-ACTIN is used as a loading control. A representative experiment of 3 is shown. Student's t test untreated cells versus TGF-β-treated cells: $**p < 0.01$; $*** p < 0.001$.

Interestingly, Hep3B, but not HepG2, cells up-regulated *NOX4* in response to TGF-β (Fig. 10A). Furthermore, TGF-β-induced cell death in Hep3B correlated with an increase of ROS production (Fig. 10B), event that was not observed in TGF-β non sensitive cell lines, such as HepG2. Finally, TGF-β-induced caspase-3 activation was ROS dependent, since the use of diphenyleneiodonium (DPI), a well-known inhibitor of NADPH oxidase and other flavoproteins, blocked it (Fig. 10C). Interestingly, PLC/PRF/5, but not SK-Hep1, cells up-regulated *NOX4* in response to TGF-β (results not shown), corroborating that sensitivity to TGF-β-induced cytotoxicity correlates with increase in *NOX4* expression in different HCC cell lines.

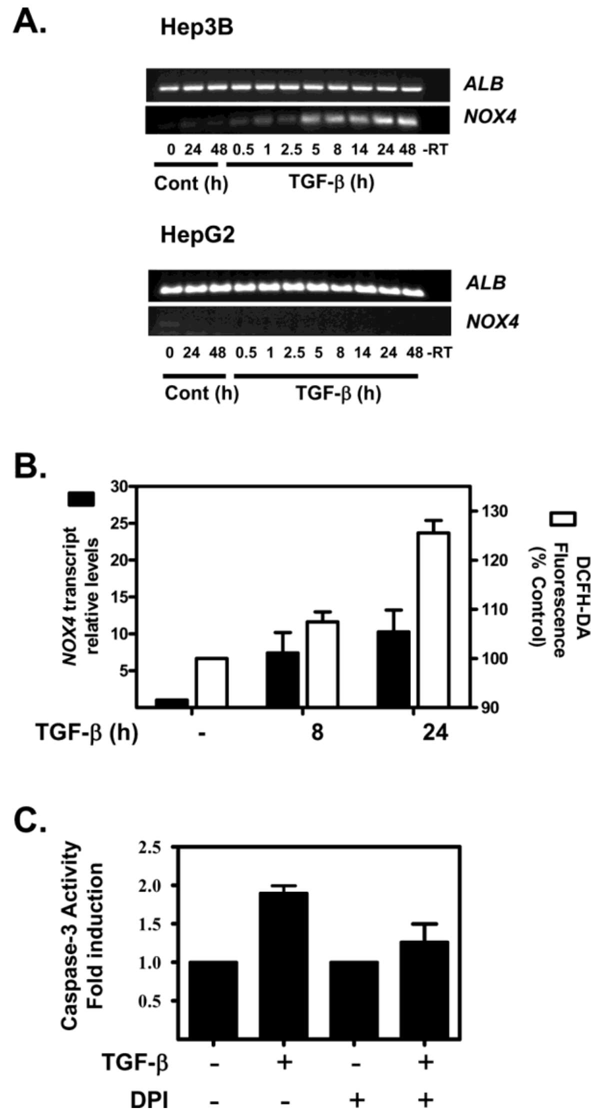


Figure 10. Up regulation of NOX4 correlates with apoptosis in TGF-β-treated hepatocellular carcinoma cell lines. A. Hep3B and HepG2 cells were incubated in the absence or in the presence of 2 ng/ml TGF-β at the indicated times. *NOX4* transcript levels were analyzed by semiquantitative PCR, *ALBUMIN* transcripts (*ALB*) are shown as control. **B.** In the case of Hep3B cells, *NOX4* up regulation was confirmed by real-time PCR and ROS production was also analyzed after TGF-β treatment at the indicated times. **C.** Caspase-3 activation was analyzed in cells treated with TGF-β with or without diphenyleneiodonium (DPI, 10 μM). Data are expressed as mean ± SEM of at least three independent experiments in duplicate/triplicate. Data from TGF-β-treated cells were compared with data from corresponding control by Student's t test (*p < 0.05; ***p < 0.001).

To analyze the relevance of NOX4 in the apoptosis induced by TGF-β in Hep3B cells, we targeted knock-down NOX4 levels by using siRNA. Hep3B cells were easily transfected, with efficiencies around 80 %, which correlated with similar knock-down in *NOX4* transcript levels and a similar decrease in TGF-β-induced ROS production (Figs. 11A-B). Under these circumstances, activation of caspase-3 and induction of cell death by TGF-β were blocked (Figs. 11C-D). These results together indicate that response to TGF-β in terms of *NOX4* up-regulation might condition the response to apoptosis in HCC cells.

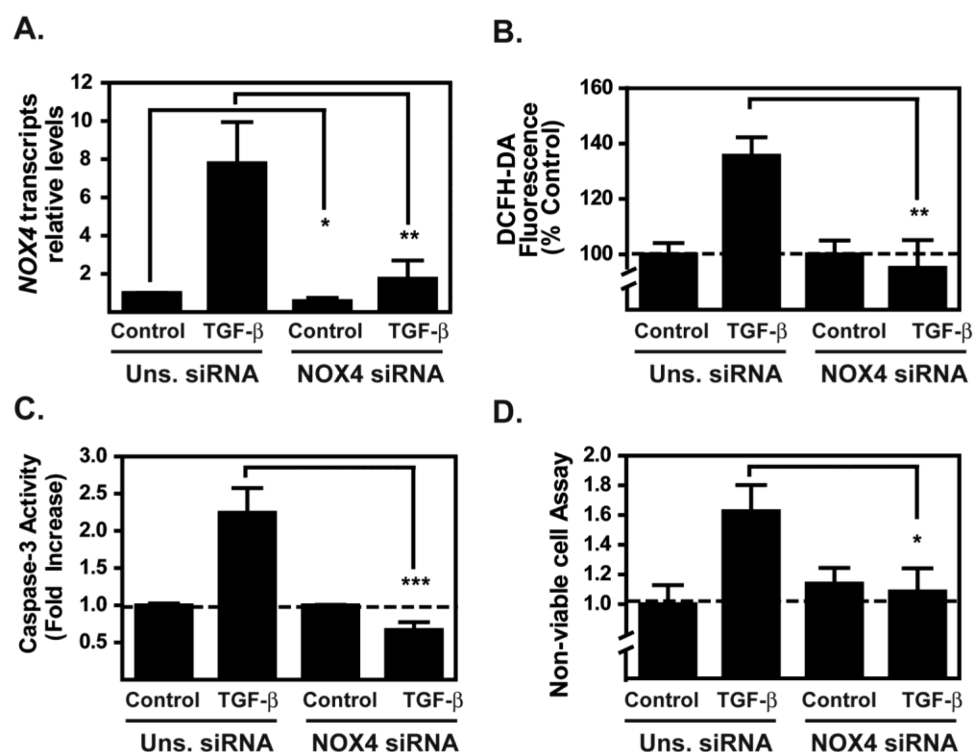


Figure 11. **siRNA targeted knock-down of NOX4 protects Hep3B cells against TGF-β-induced apoptosis**. **A.** *NOX4* transcript levels, analyzed by real-time PCR, in cells transfected with either an unspecific siRNA (Uns. siRNA) or the specific NOX4 siRNA in the absence or in the presence of TGF-β (2 ng/ml) during 36 h. **B.** Effect of the presence of NOX4 siRNA on ROS production (24 h of TGF-β treatment). **C** and **D.** Effect of the presence of NOX4 siRNA on Caspase-3 activation (16 h of TGF-β treatment) and cell death (36 h of TGF-β treatment). Data are expressed as fold-induction over the fluorescence value of control, untreated cells. Data are expressed as mean ± SEM of four independent experiments in duplicate/triplicate and compared as indicated in each figure (Student's t test: $*p < 0.05$; $**p < 0.005$).

The dual role played by TGF-β in the control of apoptotic genes in Hep3B cells (Fig. 9B) led us to study whether TGF-β would transactivate the EGFR pathway in these cells. Results indicated that TGF-β induced the gene expression of different EGFR family ligands, *TGFA*, *HB-EGF* and *Amphiregulin* (*AR*) (Fig. 12A). Furthermore, TGF-β induced the phosphorylation of SRC family after 3 hours of treatment, followed by an increase in the levels of phosphorylated forms of AKT and ERK1/2 (Fig. 12B). It is worthy to note that, similarly to that observed in FaO rat hepatoma cells, Hep3B cells showed basal levels of the EGFR family ligands *AR* and *HB-EGF*, and basal activation of the above-mentioned survival pathways (Fig. 12).

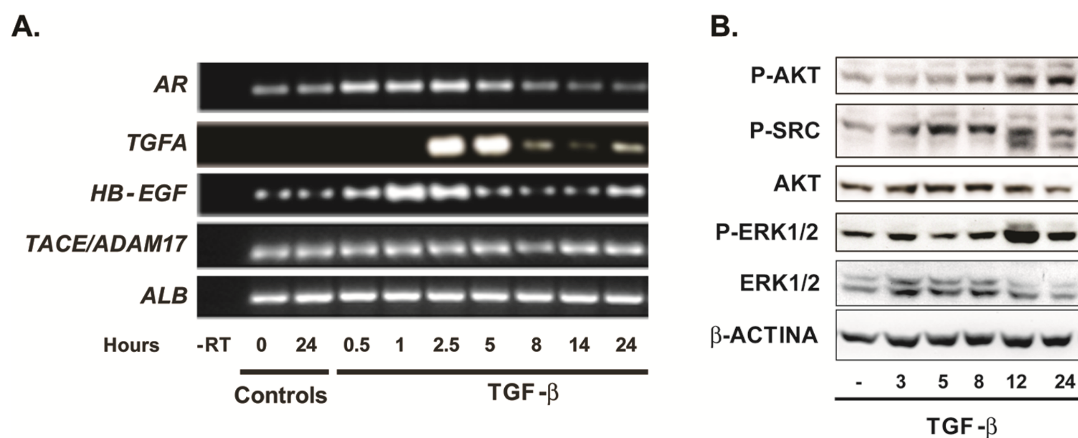


Figure 12. TGF-β activates the EGFR pathway in HCC cells. Hep3B cells were incubated with TGF-β (2 ng/ml) at the indicated times. **A.** Levels of *AR, TGFA, HB-EGF* and *TACE* transcripts were analyzed by RT-PCR. *ALB* transcripts are shown as control. **B**. TGF-β induces phosphorylation of AKT, SRC and ERKs. Western Blot analysis, β-ACTIN is used as a loading control. A representative experiment of 3 is shown.

We next decided to study the importance of the EGFR pathway in TGF-β signaling by using two different approaches: 1) EGFR silencing using siRNA specific oligos and 2) the use of different EGFR Tyrosine-Kinase activity inhibitors (AG1478 and Gefitinib).

Results indicated that EGFR silencing in Hep3B cells had consequences on culture progression after serum depletion, even in the absence of TGF-β. Indeed, targeting knock-down of the EGFR, corroborated by real time PCR and Western Blot (Figs. 13A-B), decreased the basal activation of AKT, although it had no significant effect on ERK1/2 activation (Fig. 13B). Furthermore, EGFR silencing caused a decrease in the number of viable cells, which correlated with a slight decrease in DNA synthesis, but a significant increase of caspase-3 activity (Figs. 13C-E). To confirm that a basal activation of the EGFR pathway exists in Hep3B cells, we immunoprecipitated phospho-tyrosine proteins by using an anti-phosphotyrosine antibody (clone 4G10), and we submitted them to EGFR detection by Western blot. Results indicated that serum withdrawal induced EGFR phosphorylation, although at a smaller extent than the observed by treatment with extracellular EGF (Fig. 13F).

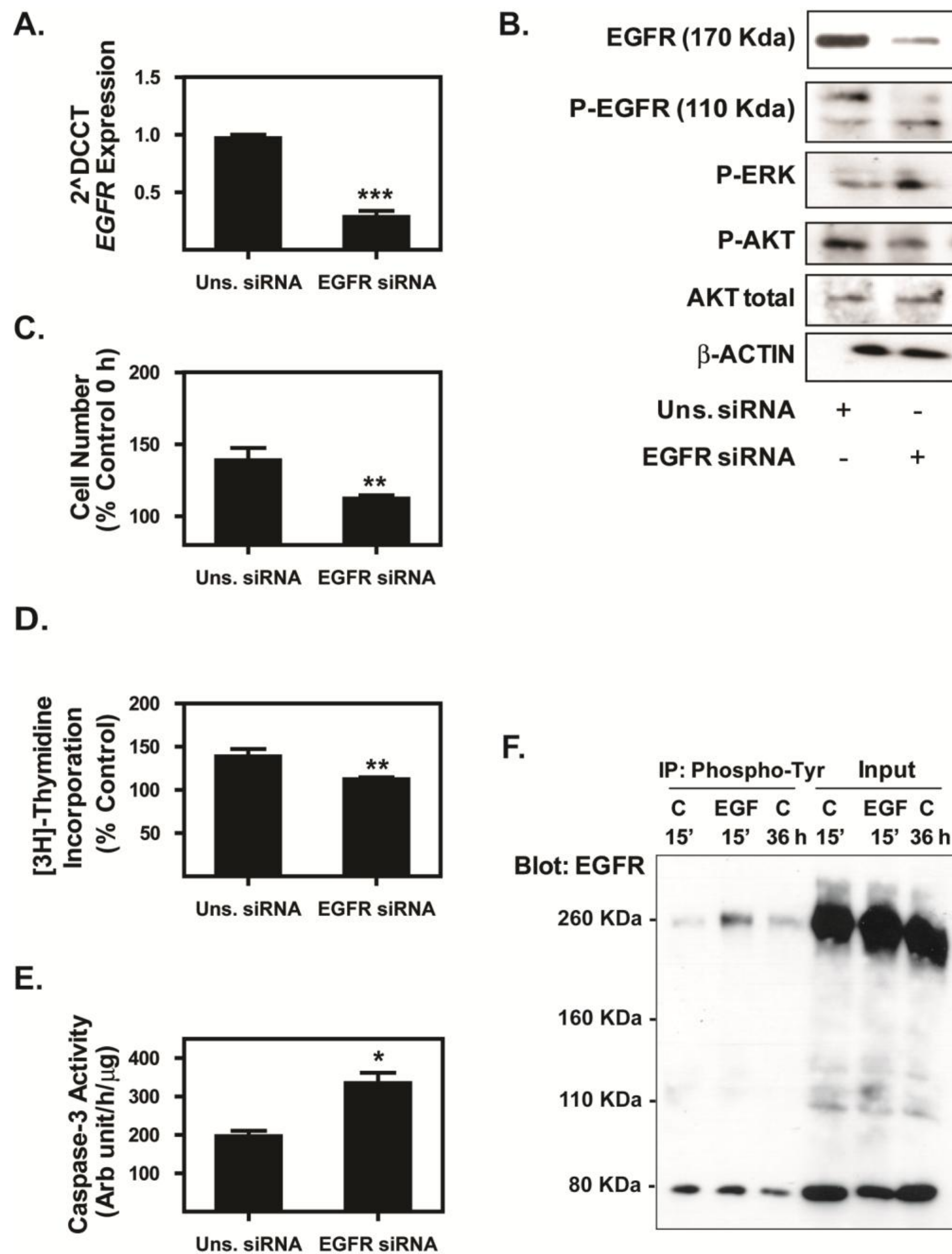


Figure 13. EGFR knock-down induces loss of cell viability. Hep3B cells were transfected with either an unsilencing (Uns) siRNA or specific siRNAs for EGFR. **A.** *EGFR* transcript levels, analyzed by real-time PCR. **B.** Effect of the presence of EGFR siRNA on autocrine-induced survival signals, Western Blot analysis, β-ACTIN is used as a loading control. **C**- **E.** Effect of the presence of EGFR siRNA after serum withdrawal on: **C**. Cell viability after 24 hours analyzed by crystal violet; **D**. [3H]-Thymidine incorporation after 48 hours; and **E**. Caspase-3 activation after 24 hours. **F.** Hep3B cells were incubated in the absence of serum with or without EGF during 15 minutes and without serum for 36 hours. Whole protein extracts were used for immunoprecipitation with anti-phospho-tyrosine antibody. EGFR was detected by Western blot. In A and C-E, data are expressed as mean ± SEM of 2-4 independent experiments in duplicate/triplicate. Effect of EGFR siRNA was compared to unsilencing siRNA (Student's t test: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

Not only EGFR knock-down had an effect on cell progression after serum withdrawal, but also EGFR silencing potentiated TGF-β effects in terms of apoptosis as it enhanced loss of cellular viability, coincident with caspase-3 activation (Figs. 14 A-B). Interestingly, *NOX4* expression induced by TGF-β after 16 hours of treatment was further up-regulated by EGFR silencing (Fig. 14C). Moreover, EGFR knock-down together with TGF-β treatment induced changes in the expression profile of BCL-2 and IAP families: up-regulation of *BMF, BIM* and *BIK*, and down-regulation of *HIAP1* were observed at the mRNA level (Fig. 15A). These changes in the expression of apoptosis-regulated proteins correlated with an increase in the percentage of cells that showed the activated form of BAX or BAK (Fig. 15B).

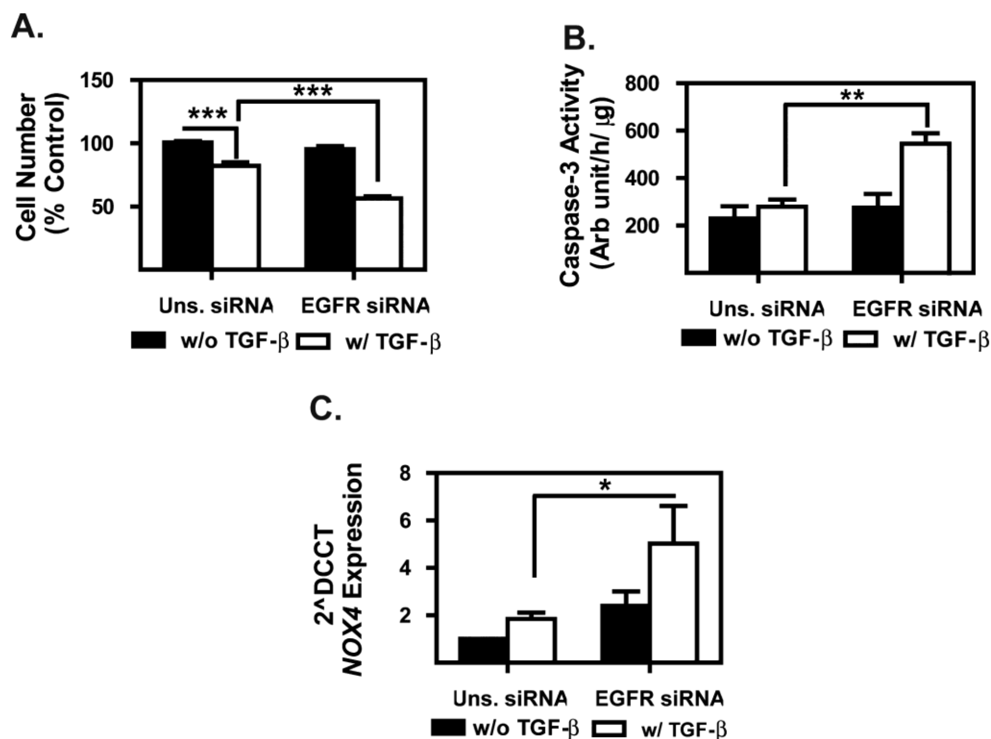


Figure 14. TGF-β-induced apoptosis in Hep3B cells is enhanced by EGFR silencing. Hep3B cells were transfected with either an unspecific siRNA (Uns. siRNA) or the specific EGFR siRNA in the absence or in the presence of TGF-β (2 ng/ml). **A.** Number of viable cells after 48 h of treatment, analysed by crystal violet staining. Results are expressed as percentage relative to the number of untreated cells. **B.** Caspase-3 activity after 16 h of treatment. **C.** *NOX4* transcript levels, analyzed by real-time PCR. Data are the mean ± S.E.M. of three different experiments in duplicate/triplicate and compared as indicated in each figure, Student's t test: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

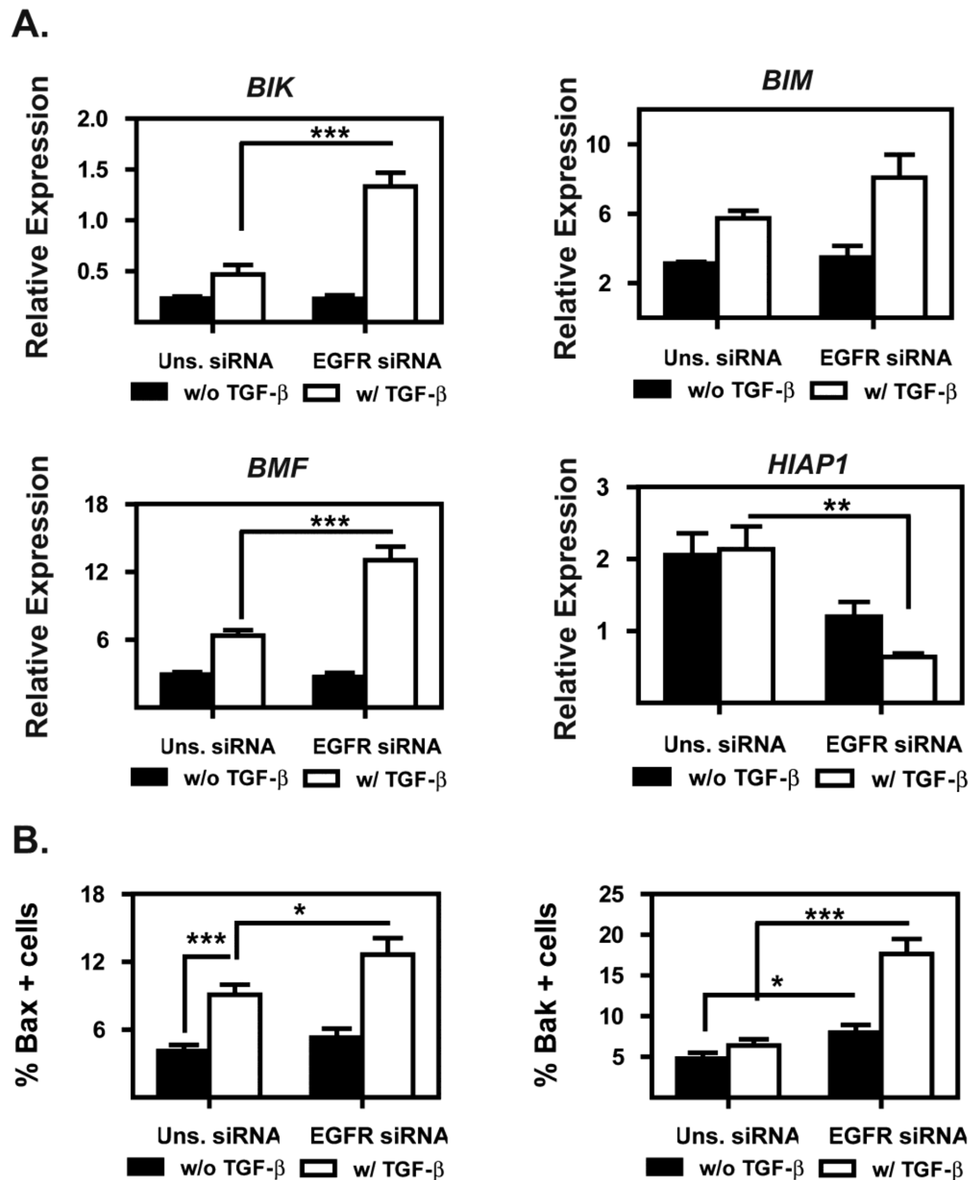


Figure 15. EGFR knockdown enhances TGF-β-induced mitochondrial apoptotic pathway. Hep3B cells were transfected with either an unspecific siRNA (Uns. siRNA) or the specific EGFR siRNA in the absence or in the presence of TGF-β (2 ng/ml) during 16 hours of treatment. **A.** Transcript levels of *BIK*, *BIM*, *BMF* and *HIAP1* analyzed by RT-MLPA. Mean ± S.E.M. of 4 different experiments, expressed as relative expression. **B.** Percentage of cells with active BAK or BAX, by immunofluorescence. Data are mean ± SEM of 3 independent experiments. Data are compared as indicated in each figure, Student's t test: *p <0.05; **p <0.01; *** p < 0.001.

The use of the thyrphostine AG1478, widely used as EGFR inhibitor, potentiated the TGF-β-induced cytotoxic effects in the TGF-β sensitive cell lines Hep3B and PLC/PRF/5 (Figs. 16A B). It is worthy to mention that AG1478 alone had an important effect on cell number in HCC cell lines, much higher than that observed after EGFR silencing. The presence of AG1478 increased TGF-β-induced activation of Caspase-3 in Hep3B cells, which correlated with a decrease in the expression of the anti-apoptotic proteins MCL1 and BCL-XL (Fig. 16C).

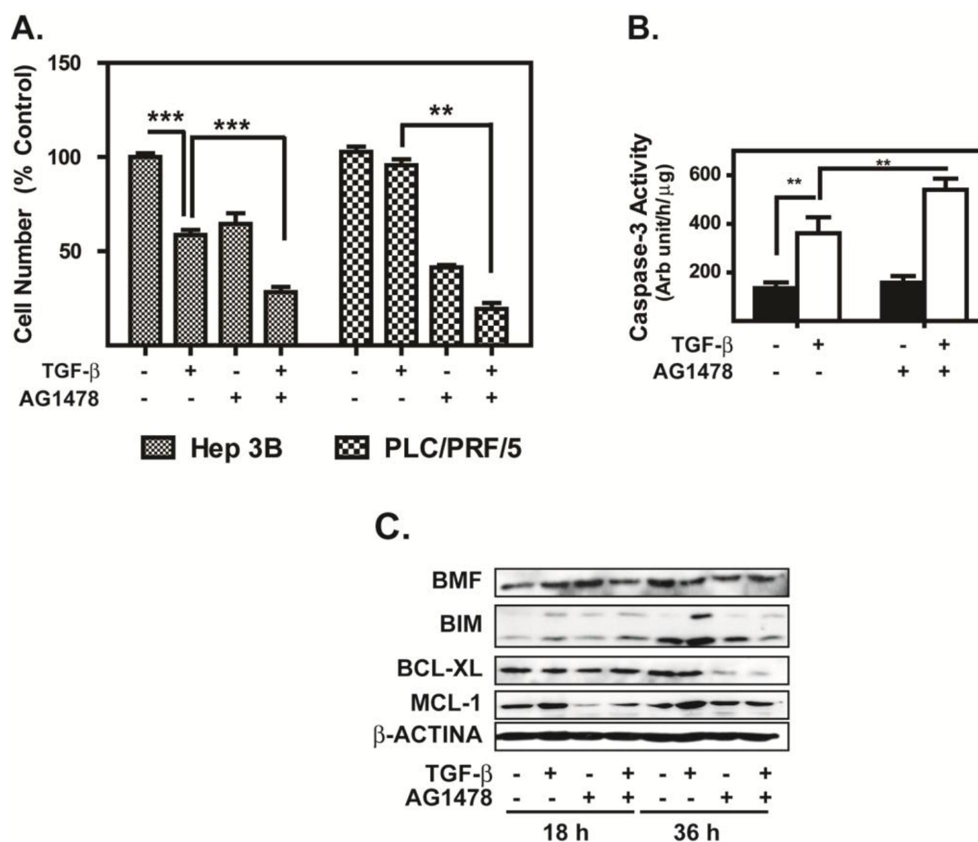


Figure 16. EGFR inhibition by AG1478 potentiates TGF-β effects in HCC cell lines sensitive to TGF-β-induced cell death. Hep3B and/or PLC/PRF/5 cells were incubated with or without TGF-β in the presence or absence AG1478 (20 μM). **A.** Number of viable cells after 48 hours of treatment, analysed by crystal violet staining. Results are expressed as percentage of the number of untreated cells. In Hep3B cells: **B.** Caspase-3 activity after 16 hours of treatment; **C.** Western blot analysis of the indicated proteins, β-ACTIN is used as loading control; a representative experiment of 3 is shown. Data are mean ± S.E.M. of three different experiments in duplicate/triplicate and compared as indicated in each figure, Student's t test: *p <0.05; **p <0.01; *** p < 0.001.

Similar results were obtained when Gefitinib was used. In Fig. 17, it is shown how Gefitinib potentiated TGF-β induced loss of viable cells in Hep3B and PLC/PRF/5 cells (Fig. 17A). In contrast, Gefitinib effects in cell viability were less potent than those observed with AG1478 alone and more similar to those shown for EGFR silencing. These results indicate that gefitinib is a more specific inhibitor, whereas AG1478 might have other targets in addition to the EGFR. The combination of TGF-β and Gefitinib potentiated TGF-β-induced caspase-3 activation and increase in the percentage of hypodiploid cells in Hep3B cells (Fig. 17B-C).

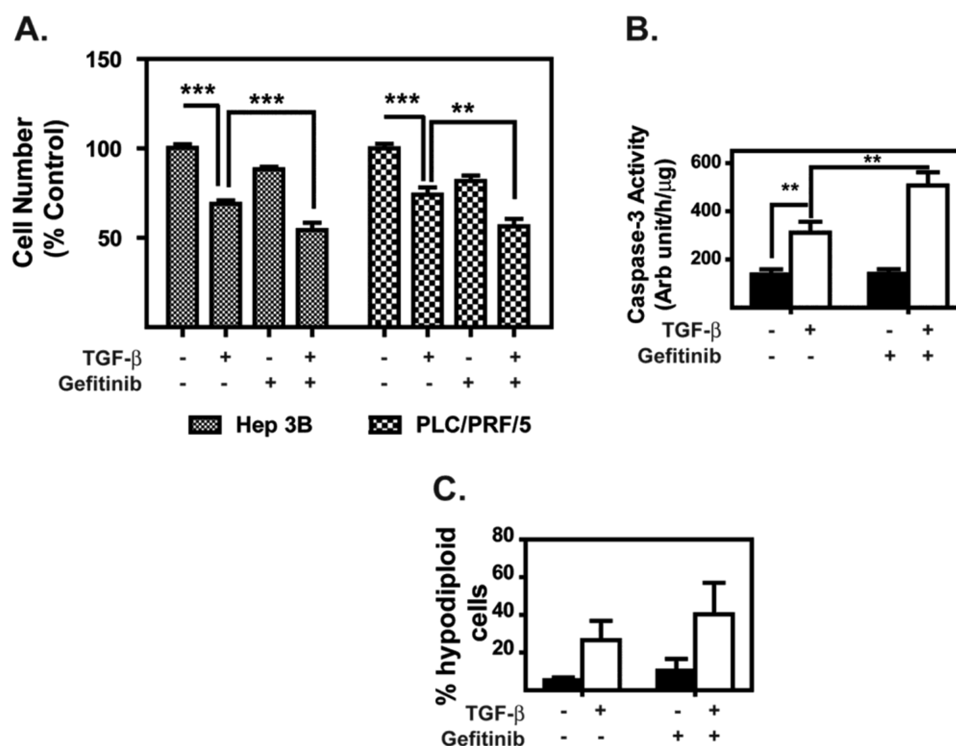


Figure 17. EGFR inhibition by Gefitinib potentiates TGF-β effects in HCC cell lines sensitive to TGF-β-induced cell death. Hep3B and/or PLC/PRF/5 cells were incubated with or without TGF-β in the presence or absence of Gefitinib (2.5 μM). **A.** Number of viable cells after 48 hours of treatment, analysed by crystal violet staining. In Hep3B: **B.** Caspase-3 activity after 16 hours of treatment and **C.** DNA content, analysed by flow cytometry, was measured after 24 h of treatment. The percentage of hypodiploid (apoptotic) cells is shown. Graphs show the mean ± S.E.M. of 2-4 different experiments in duplicate/triplicated and compared as indicated in each figure, Student's t test: $**p < 0.01$; $*** p < 0.001$.

In summary, results showed here indicate that TGF-β induces a dual response, inducing both a mitochondrial-mediated apoptosis process for which NOX4 expression is required, and survival signals through the EGFR pathway. Blockage of the EGFR results in the enhancement of TGF-β-induced apoptosis by increasing NOX4 expression and modulating TGF-β-induced changes in BCL-2 and IAP families.

2. Analysis of intracellular survival signals, independent of the EGFR pathway, that may counteract TGF-β-induced apoptosis in human hepatocarcinoma cells.

2.1. EGFR is not always responsible for acquired resistance to TGF-β-induced cell death.

Results shown above indicated that the EGFR pathway mediates the anti-apoptotic signals induced by TGF-β in HCC cells. We next wondered whether an autocrine activated EGFR pathway might be responsible for the resistance towards TGF-β cytotoxicity observed in HepG2 and SK-Hep1 cells. We first analyzed if EGFR silencing sensitized HepG2 and SK-Hep1 cells to TGF-β-induced cell death. Even though EGFR knock-down was efficient (Fig. 18A), it was unable to sensitize them to TGF-β induced loss of cell viability and activation of caspase-3 (Fig. 18B-C). Similar results were obtained when Gefitinib was used (Fig. 19).

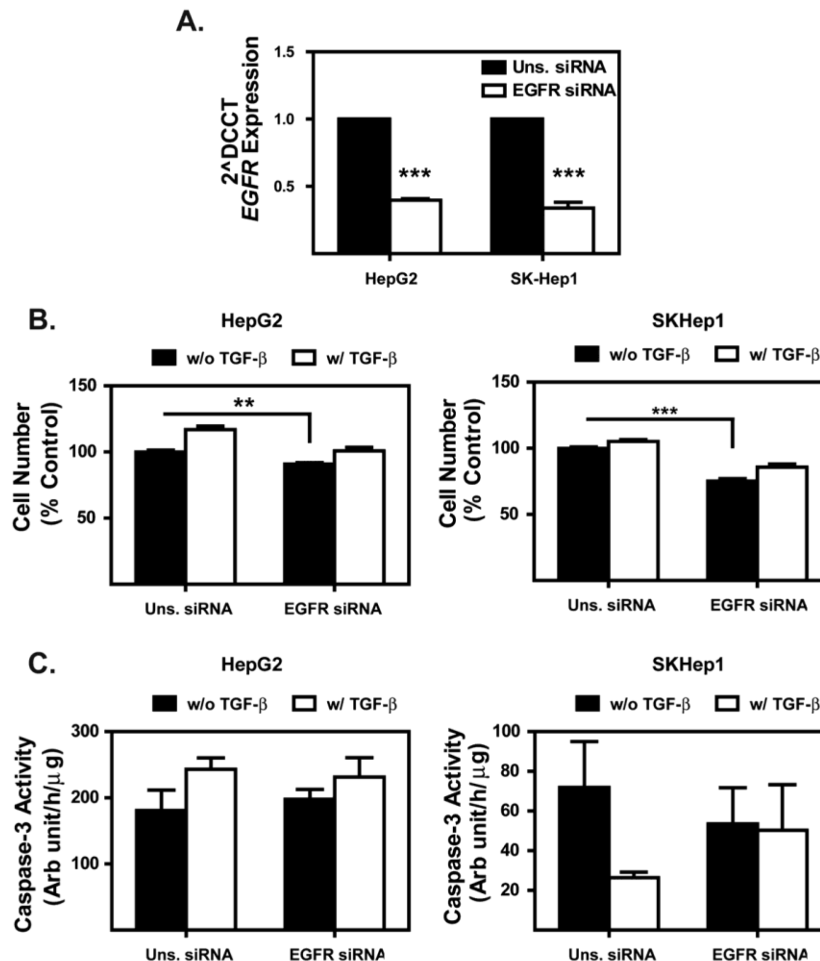


Figure 18. EGFR silencing does not sensitize HepG2 or SK-Hep1 cells to TGF-β-induced cell death. HepG2 and SK-Hep1 cells were transfected with either an unspecific siRNA (Uns. siRNA) or the specific EGFR siRNA in the absence or in the presence of TGF-β (2 ng/ml). **A.** *EGFR* expression is analyzed by real time-PCR. **B.** Number of viable cells after 48 hours of treatment, analysed by crystal violet staining. **C.** Caspase-3 activity after 16 hours of treatment. Results are the mean ± S.E.M. of three different experiments in duplicate/triplicate. Student's t test as indicated: **p <0.01 ***; p < 0.001.

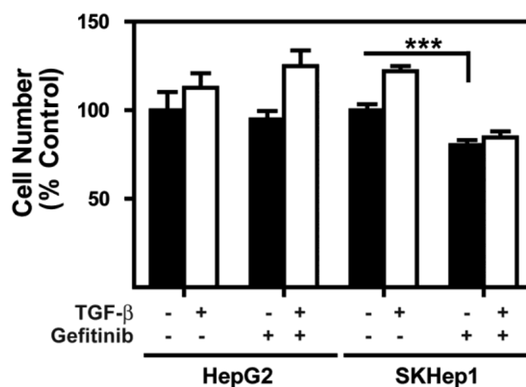


Figure 19. EGFR inhibition by Gefitinib does not sensitize HepG2 or SK-Hep1 cells to TGF-β-induced cell death. HepG2 and SK-Hep1 cells were incubated with or without TGF-β in the presence or absence of Gefitinib (2.5 μM). Number of viable cells after 48 hours of treatment, analysed by crystal violet staining. Results are the mean ± S.E.M. of three different experiments in triplicate. Student's t test as indicated: *** $p < 0.001$.

Altogether these results directed our investigation to analyze other survival pathways, independent of the EGFR activation, which might be conferring resistance to TGF-β-induced apoptosis in HCC cells.

2.2. Role of the MEK/ERK pathway in conferring resistance to TGF-β-induced cell death in HepG2 cells.

In agreement with previously shown results (Figs. 7 and 18), HepG2 cells did not respond to TGF-β in terms of apoptosis (Fig. 20A) as there was no loss in the number of viable cells and no increase in caspase-3 activity, even though these cells responded to TGF-β inducing SMAD2 phosphorylation (Fig. 20B). Among different pharmacological kinase inhibitors, we could observe that a specific MEK inhibitor, PD98059, was able to sensitize HepG2 cells to TGF-β-induced loss of cell viability (Fig. 20C). Western blot experiments revealed that HepG2 cells, cultured in the absence of FBS, showed constitutive phosphorylation of ERK (Fig. 21A). This result is in agreement with previous reports indicating that HepG2 cells show alterations in the RAS pathway, which mediates over-activation of the ERK1/2 MAPKs (Hsu et al. 1993). Other HCC cell lines that are sensitive to TGF-β-induced cell death, such as PLC/PRF/5 and Hep3B, showed much lower levels of phospho-ERKs activation. A correlation was found between ERK activation and resistance to cell death (Fig. 21A), as the lower ERK phosphorylation is, the greater TGF-β cytotoxic effect is. Interestingly, inhibition with PD98059 enhanced the cytotoxic response to TGF-β in both PLC/PRF/5 and Hep3B cells (Fig. 21B-C). Analysis of caspase-3 in other HCC cells (Hep3B cell line) confirmed that MEK inhibition was enhancing TGF-β-induced apoptosis (Fig. 21D). However, MEK pathway inhibition in SK-Hep1 cells did not sensitize these cells to TGF-β cytotoxicity (results not shown), which indicate that other molecular mechanisms must be responsible for resistance to TGF-β in these cells.

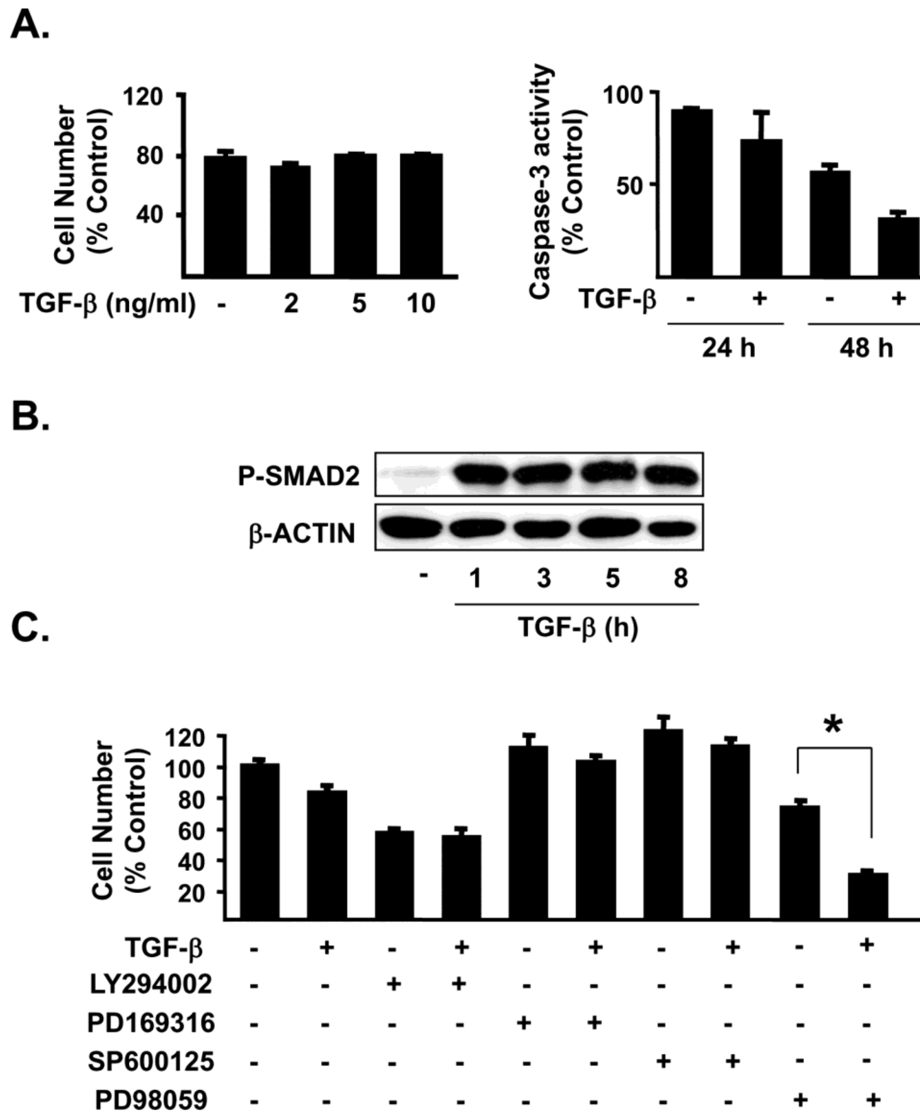


Figure 20. The MEK inhibitor PD98059 sensitizes HepG2 cells to TGF-β-induced cell death. A. Cell viability after 72 h of treatment with TGF-β at different concentrations was analyzed by Cristal Violet. **B.** Western blot analysis of the levels of phospho-SMAD2 after TGF-β treatment. β-ACTIN was used as loading control. **C**. Cell viability, analyzed as described in A, after 48 h of treatment with TGF-β and different protein kinase inhibitors: PI3K (LY294002, 5 μM), p38MAPK (PD169316, 800 nM), JNK (SP600125, 40 μM) and MEK (PD98059, 50 μM). **A** and **C:** Mean ± SEM of 3 independent experiments, expressed as percentage of untreated cells. **B:** A representative experiment (n=3). Student's t test as indicated: * $p < 0.05$.

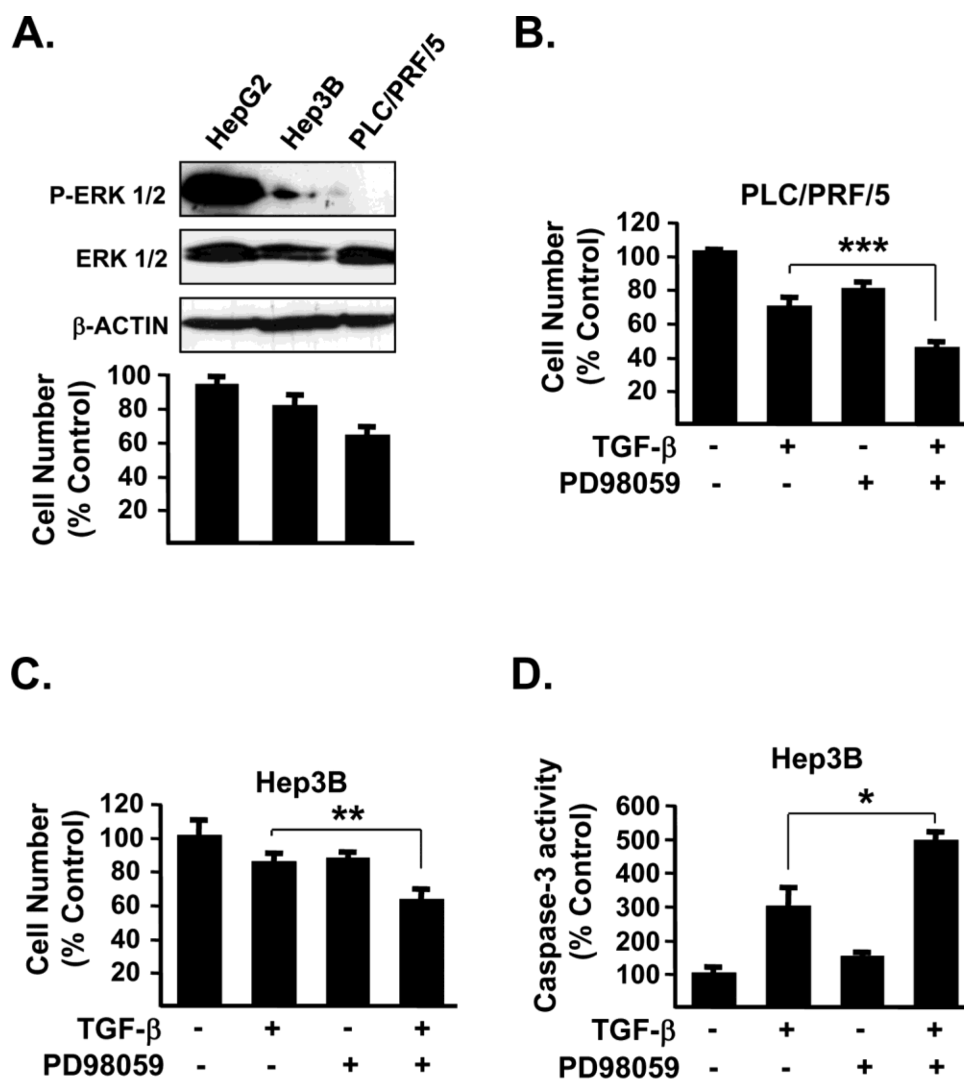


Figure 21. Response of different HCC cells to TGF-β. Correlation with ERKs activation. Effect of the MEK inhibitor PD98059. A. Top. Analysis of the levels of phospho-ERKs by Western blot in HepG2, Hep3B and PLC/PRF/5 cells (representative experiment: n=3, β-Actin is used as a loading control, cells cultured in the absence of FBS for 8 h). **Bottom**: Cell viability analyzed by Cristal Violet after 48 h of treatment in the presence of TGF-β (2 ng/ml) and expressed as percentage of untreated cells. **B, C**. Cell viability after 48 h of treatment in the absence or the presence of TGF-β (2 ng/ml) with or without the MEK inhibitor PD98059 (50 μM) analyzed by Cristal Violet in both PLC/PRF-5 cells (**B**) or Hep3B cells (**C**). **D**. Caspase-3 activity in Hep3B cells treated for 16 h as indicated in B. Results are the mean ± S.E.M. of 3 different experiments and are expressed as percentage of untreated cells. Data are compared as indicated in each figure, Student's t test t: *p <0.05; **p < 0.01; *** p < 0.001.

To understand the mechanism by which MEK inhibition restored TGF-β sensitivity in HepG2 cells, first we analyzed whether activation of ERKs might alter the TGF-β canonical pathway. We could not observe significant differences either in SMAD2 phosphorylation, or in the expression levels of different *SMADs* (included the inhibitory *SMAD6* and *SMAD7*) and *TGF-β Receptors I* and *II*, when the MEK/ERK pathway was inhibited in HepG2 cells (Figs. 22A-B). This result suggested that sensitization of HepG2 cells to TGF-β-induced cell death might occur downstream from TGFβ-receptors/SMADs activation. Levels of p53 did not significantly changed after cell treatment with TGF-β, neither with PD98059 nor the combined treatment, excluding that MEK inhibition might affect the regulation of TGF-β on this pathway (Fig. 22C).

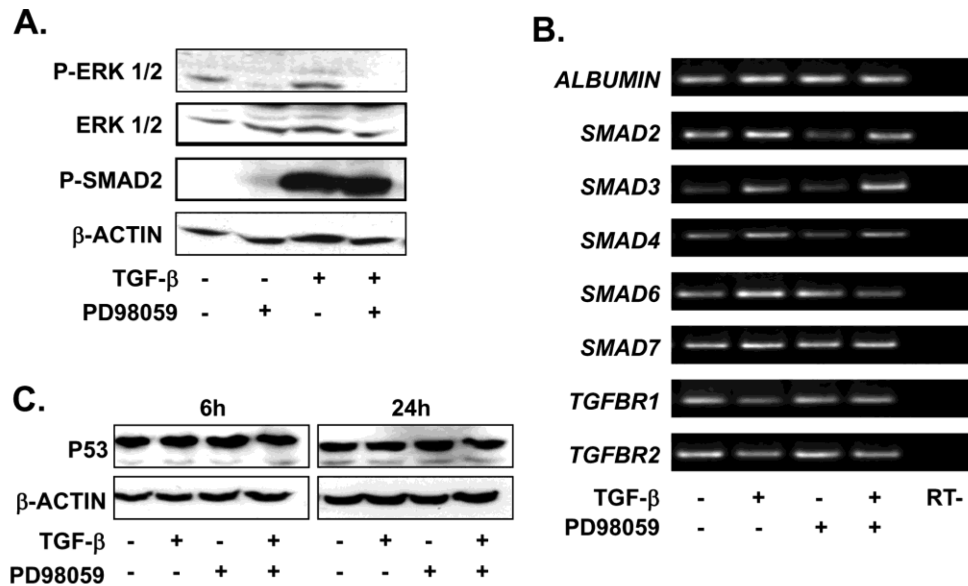


Figure 22. The MEK inhibitor PD98059 has no effect on SMAD2 phosphorylation, expression of SMADs and TGF-β Receptors (I and II), or p53 levels. HepG2 cells were incubated in the absence or the presence of TGF-β (2 ng/ml) with or without the MEK inhibitor PD98059 (50 μM), as indicated in each case. **A.** ERK and SMAD2 phosphorylation after 3 hours of treatment, Western blot analysis. β-ACTIN is used as loading control. **B**. Transcript levels of *SMADs* and *TGF-β receptors I* and *II* were analyzed by RT-PCR after cells were treated for 24 h. *ALBUMIN* was used as loading control. A representative experiment (n=2). **C**. p53 levels after 6 and 24 h of treatment. Western blot analysis, β-ACTIN is used as loading control. A representative experiment (n=3).

Exploring the potential mechanism of cell death induced by the combined treatment of TGF-β+PD98059 in HepG2 cells we found a significant increase in caspase-3 activity and DNA fragmentation, analysed as the percentage of hypodiploid cells (Figs. 23A-B). All these events were coincident with loss of the mitochondrial transmembrane potential and the lysosomal membrane integrity, analyzed with specific fluorescent probes (Fig. 23C), indicating that these organelles might play a role in the mechanism of cell death. PD98059 is a specific MEK inhibitor; however, side-effects could not be excluded. Therefore, we performed controls with other MEK inhibitors, U0126 and PD0325901, and confirmed our results in terms of loss of cellular viability and caspase-3 activation (Fig. 23D). Moreover, the use of specific siRNA against ERK1 and ERK2, which down-regulated up to 70 % their expression (Fig. 24A), sensitized HepG2 cells to TGF-β-induced cell death (Figs. 24B-C). In all cases ERK inhibition sensitized HepG2 cells to TGF-β-induced loss in viability and activation of caspase-3, as a key regulator of apoptosis.

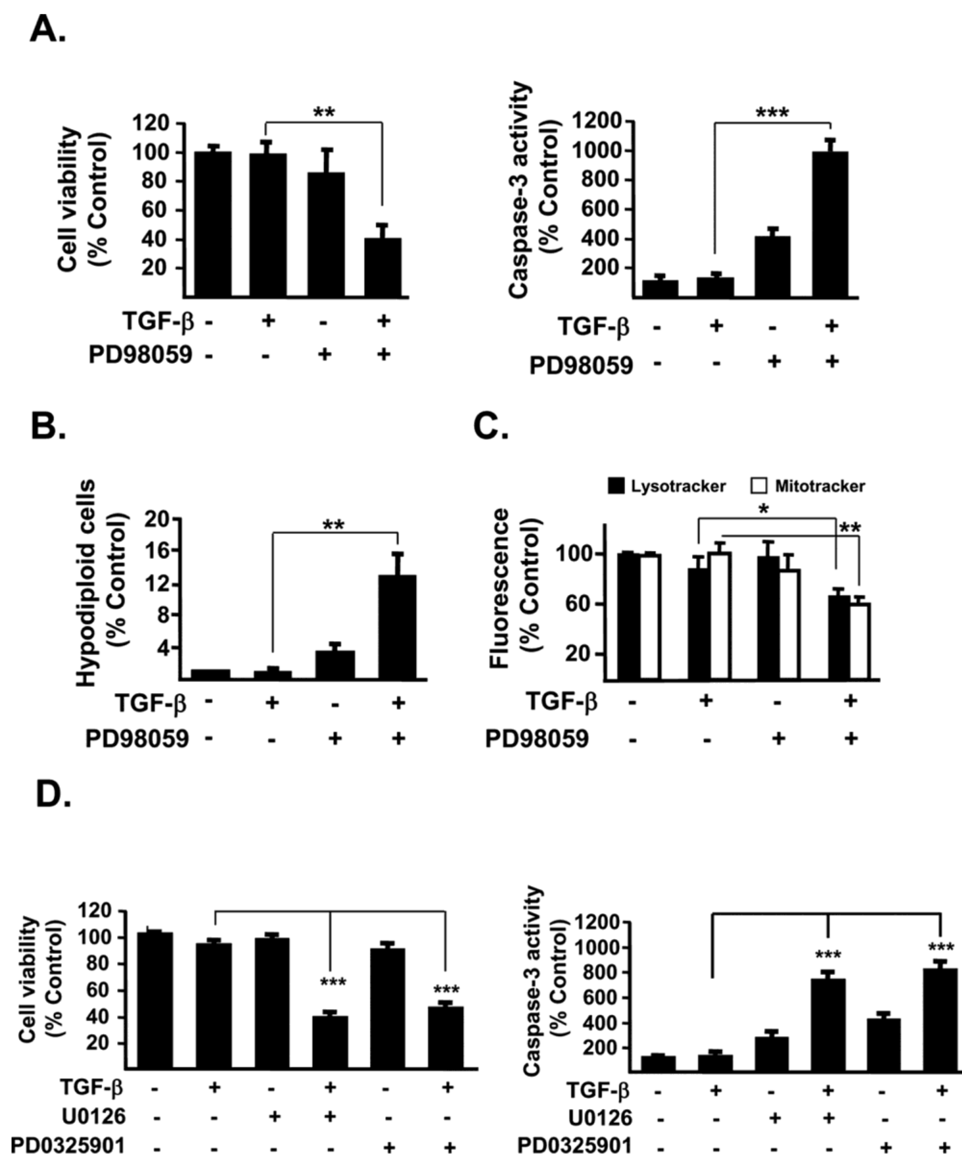


Figure 23. MEK inhibition sensitizes HepG2 cells to TGF-β-induced apoptosis. Cells were incubated in the absence or the presence of TGF-β (2 ng/ml) with or without the MEK inhibitor PD98059 (50 μM). **A. Left.** Cellular viability (24 h of treatment). **Right.** Caspase-3 activity (16 h of treatment). **B.** DNA content, analysed by flow cytometry after 24 h treatment. The percentage of hypodiploid (apoptotic) cells is shown. **C.** Analysis of lysosomal permeabilization (lysotracker) and mitochondrial transmembrane potential (mitotracker) (6 hours of treatment). **D**. Cells were incubated in the absence or the presence of TGF-β (2 ng/ml) with or without the MEK inhibitors U0126 (10 μM) or PD0325901 (2 μM), as indicated in each case. **Left,** Cellular viability (24 h of treatment). **Right,** Caspase-3 activity (16 h of treatment). In all cases, bars show the mean ± S.E.M. of 3-6 different experiments and are expressed as % of untreated cells. Data are compared as indicated in each figure, Student's t test t: *p <0.05; **p < 0.01; *** p < 0.001.

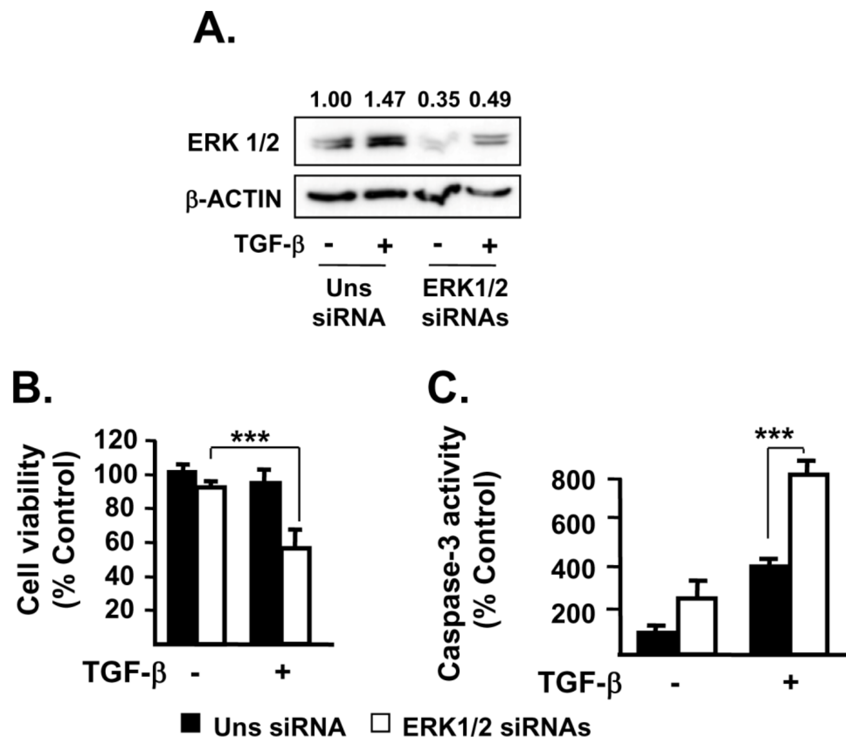
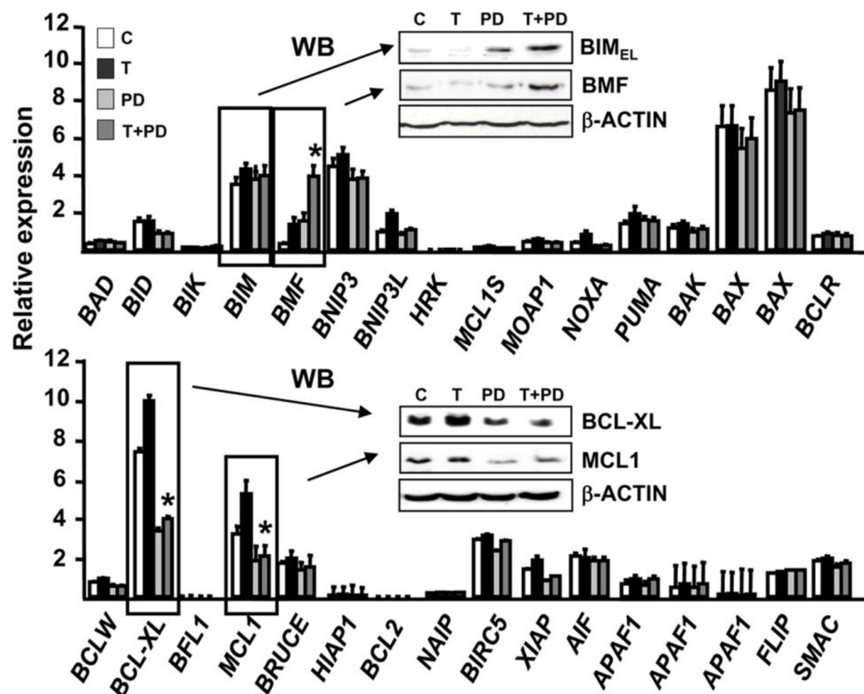


Figure 24. Effect of ERK1/2 knock-down on the response of HepG2 cells to TGF-β. HepG2 cells were transfected with either an unsilencing (Uns) siRNA or specific siRNAs for ERK1 and ERK2 and were treated in the absence or in the presence of TGF-β. **A**. Protein levels indicating the efficiency of targeting (16 h treatment with TGF-β). Numbers indicate densitometric analysis relative to β-ACTIN levels. A representative experiment (n=3). **B**. Cellular viability (24 h of treatment). **C**. Caspase-3 activity (16 h of treatment). Results are the mean ± S.E.M. of 3 different experiments and are expressed as % of untreated cells. Data are compared as indicated in each figure, Student's t test t: *** $p < 0.001$.

We next decided to focus on the molecular mechanism that might explain at which level ERK pathway is blocking TGF-β-induced apoptosis. Changes in the expression gene profile of apoptotic regulators, by using RT-MLPA, is shown in Fig. 25A. Transcript and protein levels of the BH3-only gene *BMF* were significantly enhanced with the combined treatment of TGF-β+PD98059. TGF-β induced *BCL-XL* and *MCL1*expression, both anti-apoptotic members of the BCL-2 family. However, a significant decrease in the expression of these genes was observed when the MEK/ERK inhibitor was present, which correlated with lower protein levels (Fig. 25A). Although we could not find variations in the expression of *BIM* at the mRNA levels, we found an increase in its protein levels when MEK/ERK was inhibited, effect that was significantly enhanced in the presence of TGF-β. In summary, the combined treatment of TGF-β and PD98059 produced an increase in the levels of BIM and BMF and a decrease in BCL-XL and MCL1. This expression pattern correlated with a significant enhancement in the percentage of cells showing the active conformational form of BAX or BAK (Fig. 25B).

A.



B.

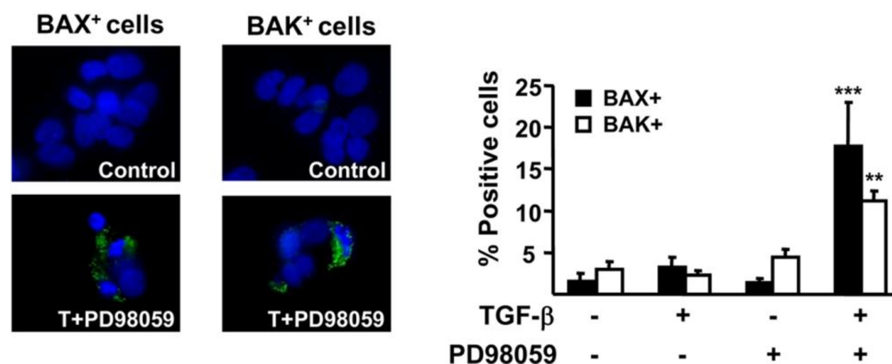


Figure 25. Combined treatment with TGF-β and PD98059 modulates the expression of different BCL-2 family members. HepG2 cells were incubated in the absence or the presence of TGF-β (2 ng/ml) with or without the MEK inhibitor PD98059 (50 μM) during 16 h. **A.** Transcript levels of apoptotic genes by RT-MLPA. Mean ± S.E.M. of 6 different experiments, expressed as relative expression. WB: Western blot analysis of the indicated genes. β-ACTIN is used as loading control, a representative experiment of 3 is shown. **B.** Analysis of BAK and BAX activation by immunofluorescence. Left: representative photographs. Right: Mean ± S.E.M. of 3 different experiments. Student's t test TGF-β+PD98059 treated cells versus TGF-β-treated cells: *p <0.05; **p < 0.01; *** p < 0.001.

2.3. Sustained oxidative stress and significant induction of the NADPH oxidase NOX4 in TGF-β-treated HepG2 cells is observed when the MEK/ERK pathway is inhibited.

In HepG2 cells, TGF-β alone was unable to induce an increase in ROS production, as analyzed with the H_2-DCFDA fluorescent probe. The inhibition of MEK/ERK pathway induced a transient, although significant, increase in the intracellular ROS content, but it was the combined treatment of TGF-β and PD98059 which produced a higher and sustained intracellular ROS increase at all the examined times (Fig. 26A). ROS increase correlated with depletion of glutathione (GSH) content, which only was observed in TGF-β+PD98059-treated cells (Fig. 26B). To evaluate the importance of this increase of ROS in the mechanism of cell death, cells were pre-incubated with a membrane-permeable form of GSH (Glutathione-ethyl-ester, GEE), which partially blocked the apoptotic events (Fig. 26C), as it decreased the activation of caspase-3 and the percentage of hypodiploid cells induced by the co-treatment with TGF-β + PD98059.

We next wondered whether MEK/ERK inhibition might confer to HepG2 cells the capacity to respond to TGF-β in terms of NOX4 up-regulation. Results indicated that TGF-β only increased *NOX4* transcript levels in HepG2 cells when MEK was inhibited, which was coincident with the maximum expression of the NOX4 co-activator *p22-PHOX* (Fig. 26D, left). Interestingly, at the protein level, we observed a slight increase in NOX4 in cells treated only with PD98059, but the highest levels were always observed with the combined treatment of TGF-β and PD98059 (Fig. 26D, right). It has been recently suggested that ERK activation is required for the induction of the antioxidant gene heme oxygenase-1 (HO-1) by TGF-β (Churchman et al. 2009). The analysis of the levels of HO-1 revealed that TGF-β induced its expression in HepG2 cells and MEK inhibition attenuated this response (Fig. 27A). Indeed, MEK inhibition sensitizes cells to respond to TGF-β up-regulating NOX4, but also impairs the up-regulation of the antioxidant gene HO-1. Other antioxidant proteins, such as Catalase or Mn-superoxide dismutase (SOD2) did not show significant changes in their levels. Interestingly, the γ-glutamylcisteine synthetase (γ-GCS) levels decreased, which might be due to caspase-3 proteolysis (Fig. 27B), as it has been described as a caspase substrate (Franklin et al. 2003).

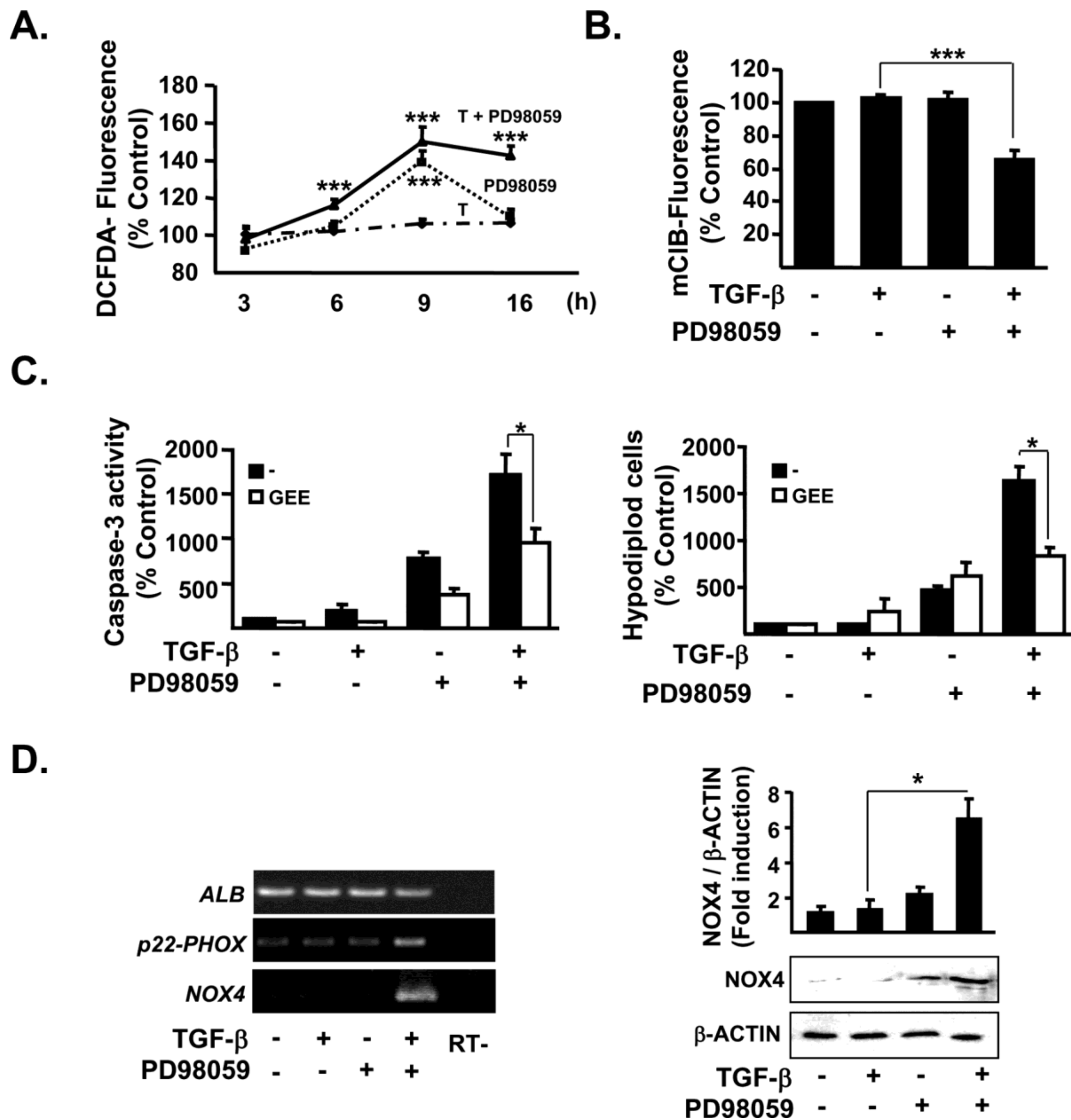


Figure 26. Combined treatment with TGF-β and PD98059 induces oxidative stress, which correlates with NOX4 up-regulation. Cells were incubated in the absence or the presence of TGF-β (2 ng/ml) with or without the MEK inhibitor PD98059 (50 μM). **A.** Intracellular peroxide content at the indicated times of treatment. **B.** Fluorimetric analysis of intracellular GSH levels (16h of treatment). **C.** Pretreatment with Glutathione ethyl ester (GEE) attenuates caspase-3 activation (16 h of treatment, Left) and analysis of the percentage of hypodiploid (apoptotic) cells, analysed by flow cytometry (24 h of treatment, Right). Mean ± S.E.M. of 3 independent experiments. **D.** Left**,** *NOX4* and *p22-PHOX* transcript levels were analyzed by RT-PCR (3 h of treatment). *ALBUMIN* (ALB) is shown as loading control. A representative experiment is shown (n=3). Right, NOX4 protein levels analyzed by Western blot (3 h of treatment). β-ACTIN is used as loading control. Densitometric analysis of 3 independent experiments is represented in the graph (mean ± S.E.M.). Data are compared as indicated in each figure, Student's t test: *p <0.05; *** p < 0.001.

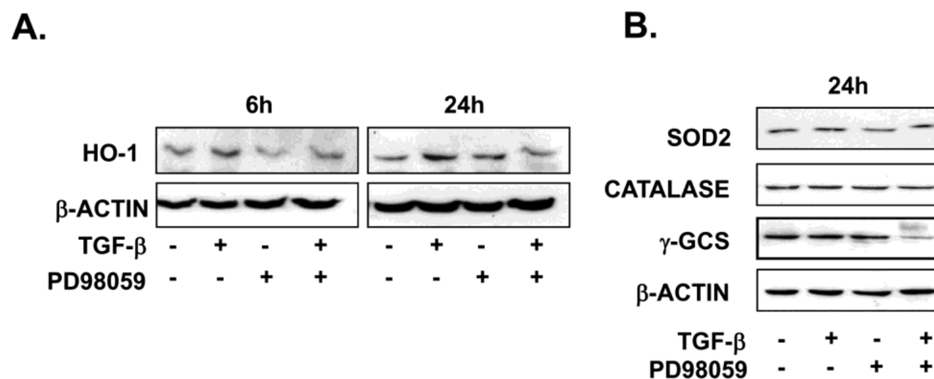


Figure 27. Combined treatment with TGF-β and PD98059 counteracts TGF-β-induced HO-1 up-regulation and produces loss in γ-GCS levels. Cells were incubated in the absence or the presence of TGF-β (2 ng/ml) with or without the MEK inhibitor PD98059 (50 μM), at the indicated times. **A**. Heme oxygenase-1 (HO-1) levels analyzed by Western blot (similar results were obtained at shorter times: 1.5, 3 h). **B**. Analysis by Western blot of other antioxidant protein. β-ACTIN was used as loading control. A representative experiment (n=3).

2.4. Role of NOX4 in the apoptosis induced by the combined treatment of TGF-β and the MEK/ERK inhibitor.

In view of these results, we had a privileged situation in which to examine the specific role of NOX4 in the apoptosis induced by TGF-β in liver cells. TGF-β-induced apoptosis switch is "off" in HepG2 cells due to the high phospho-ERKs levels, but it is "on" when MEK/ERK pathway is inhibited, coincident with NOX4 up-regulation. We decided to target knock-down NOX4 in cells, by using a siRNA approach, to specifically analyze its effect on all the cell death features. NOX4 knock-down (Fig. 28A) decreased ROS production (Fig. 28B), blocked disruption of mitochondrial transmembrane potential (Fig. 28C, Left) and significantly attenuated caspase-3 activation and the loss in cell viability induced by TGF-β + PD98059 (Fig. 28D). However, NOX4 knock-down did not attenuate the loss in lysosomal membrane integrity (Fig. 28C, Right), which suggested that NOX4 was only affecting the mitochondrial-mediated mechanism of cell death. In this same line of evidence, NOX4 knock-down attenuated TGF-β + PD98059 regulation of *BMF*, *BCL-XL* and *MCL1* at the mRNA (Fig. 29A) and protein (results not shown) levels, and BIM at the protein level (Fig. 29B). Correlating with these results, NOX4 knock-down diminished the percentage of cells showing active BAX or BAK (Fig. 29C), suggesting that NOX4 is required for TGF-β-induced mitochondrial-dependent apoptosis upstream from the regulation of BCL-2 family expression. Along the same line of evidence, pre-treatment of cells with a NADPH oxidase inhibitor, diphenyleneiodonium (DPI), alone or with GEE, completely blocked increase in ROS, caspase-3 activation and regulation of the BCL-2 family genes after TGF-β+PD98059 treatment (Figs. 30A-C).

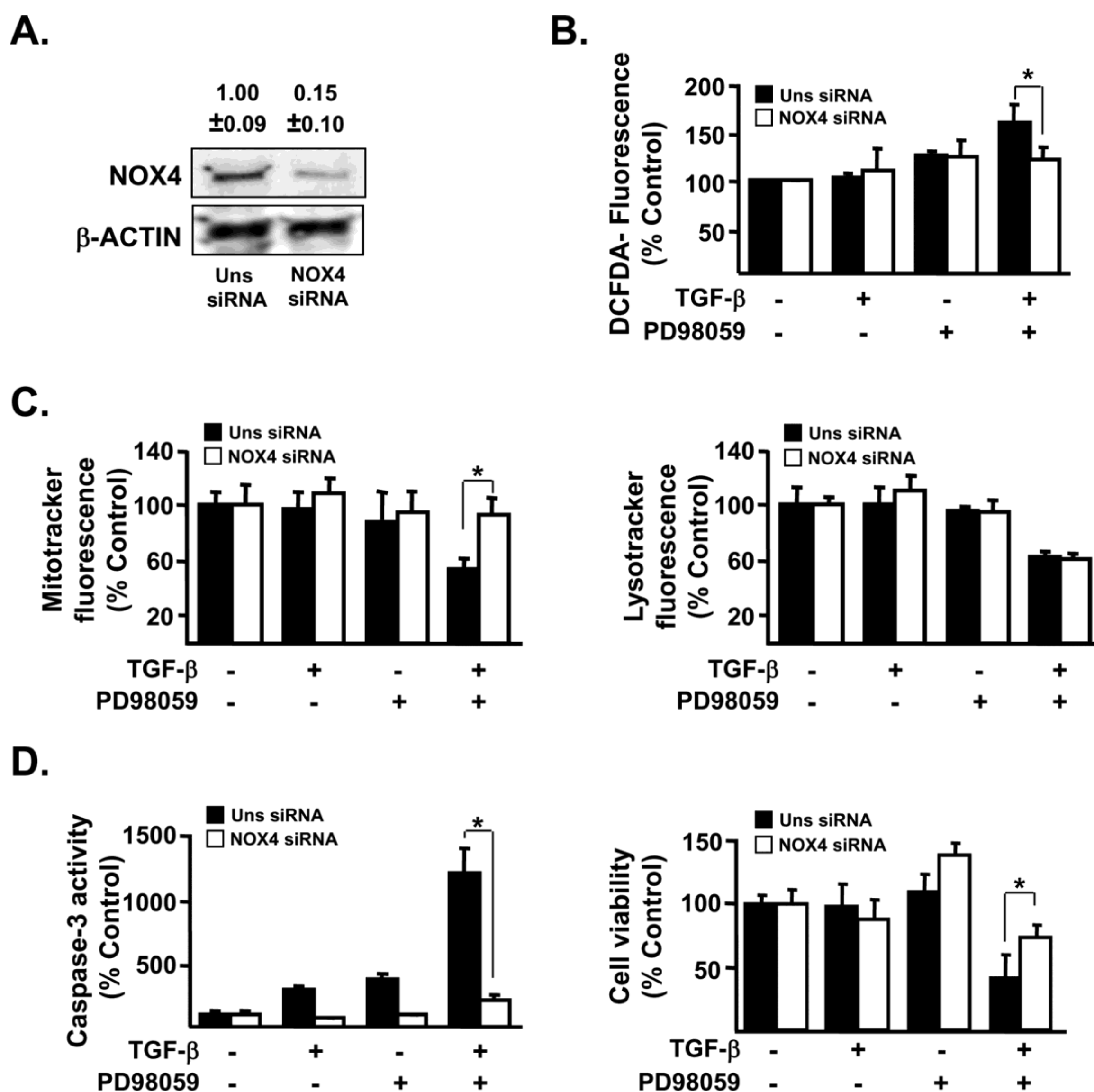


Figure 28. NOX4 targeting knock-down inhibits the mitochondrial-dependent apoptosis induced by TGF-β + PD98059. HepG2 cells transfected with either an unsilencing (Uns) siRNA or the specific NOX4 siRNA were incubated in the absence or the presence of TGF-β (2 ng/ml) with or without the MEK inhibitor PD98059 (50 μM). **A.** NOX4 protein levels, after 3 hours of cell treatment with the combination of TGF-β + PD98059. β-ACTIN is used as loading control. A representative experiment is shown. Mean ± S.E.M of densitometric analysis of 3 independent experiments is shown above the Western blot. **B.** Intracellular peroxide content after 16 h of treatment. **C.** Mitochondrial (Left) and lysosomal (Right) permeabilization, after 6 h of treatment. **D.** Caspase-3 activity after 16 h of treatment (Left), and cell viability after 24 h of treatment (Right). Data are calculated relative to unsilencing siRNA-transfected untreated cells and represented as the mean ± S.E.M of 3 independent experiments in duplicate/triplicate. Data are compared as indicated in each figure, Student's t test: *$p<0.05$.

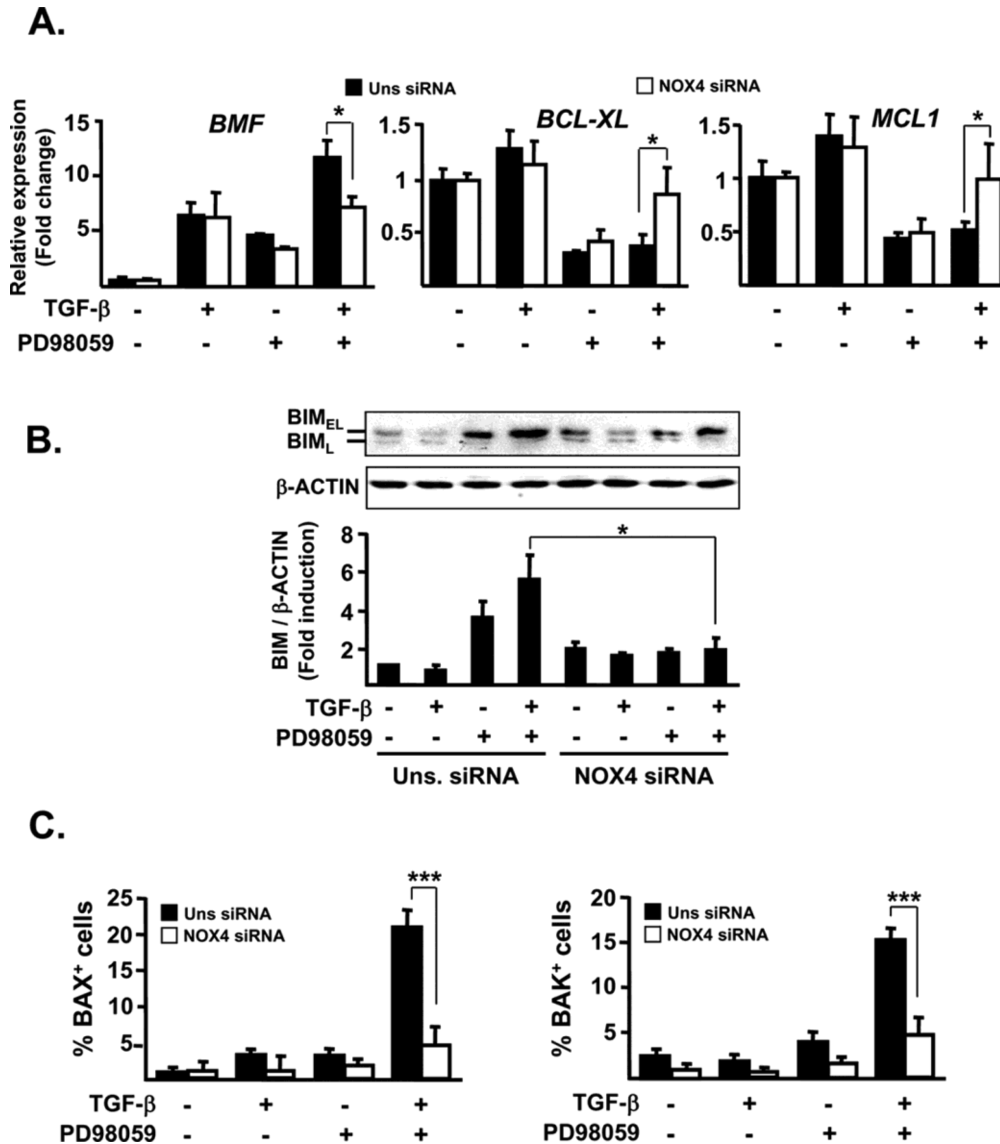


Figure 29. NOX4 targeting knock-down impairs the regulation of BCL-2 family members induced by TGF-β + PD98059. HepG2 cells transfected with either an unsilencing (Uns) siRNA or the specific NOX4 siRNA were incubated in the absence or the presence of TGF-β (2 ng/ml) with or without the MEK inhibitor PD98059 (50 μM) during 16 hours. **A**. Transcript levels of *BMF*, *MCL1* and *BCL-XL* analyzed by RT-MLPA, results are expressed as fold change relative to untreated cells.. **B.** Analysis of *BIM* at the protein level by Western Blot, β-ACTIN is used as loading control. Up: Representative experiment. Bottom: densitometric analysis, mean ± S.E.M. **C.** Percentage of cells with active BAK or BAX, analyzed by immunofluorescence. Data are calculated relative to unsilencing siRNA-transfected untreated cells and represented as the mean ± SEM of 3 independent experiments. Data are compared as indicated in each figure, Student's t test: *p <0.05; *** p < 0.001.

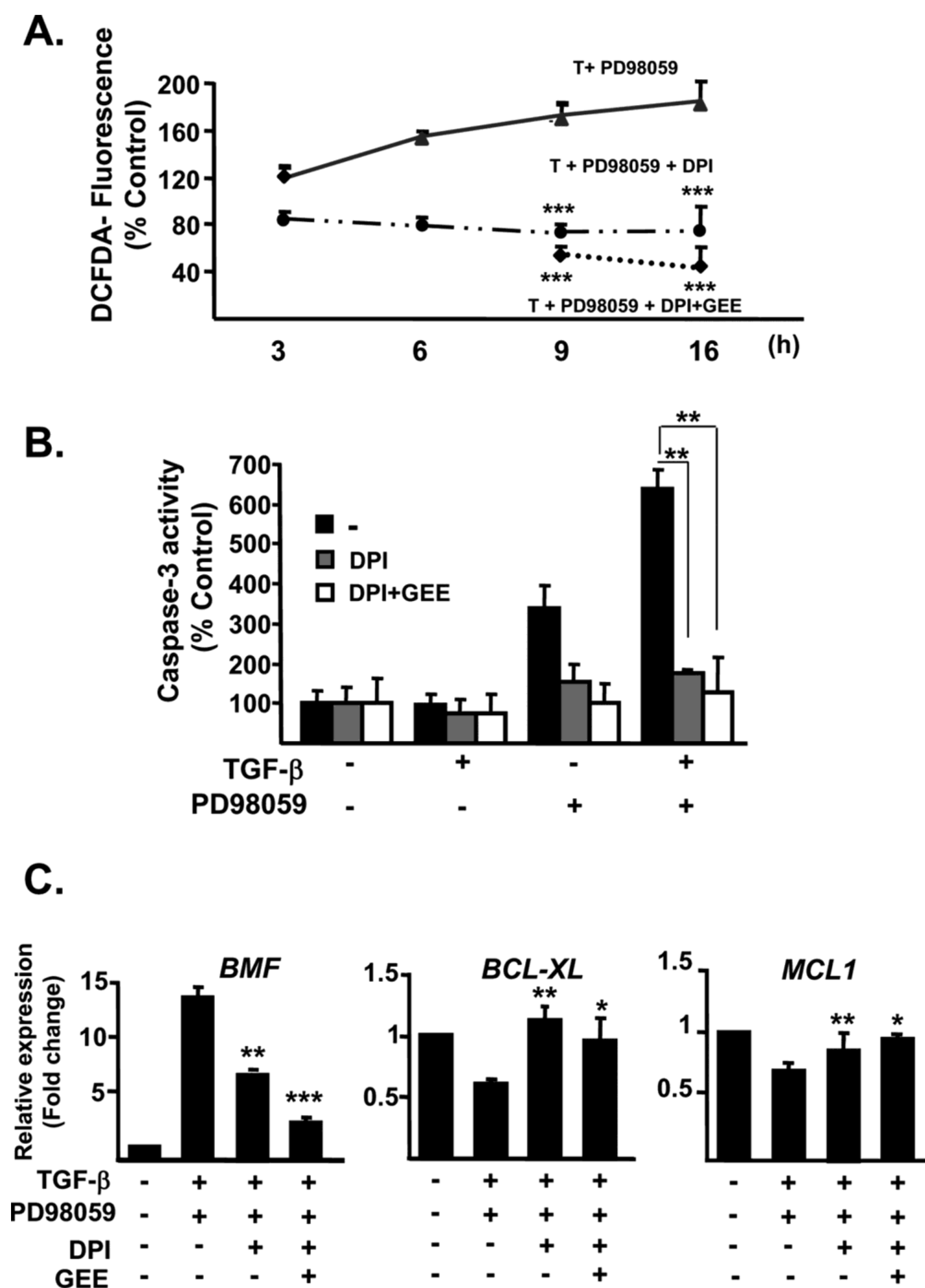


Figure 30. Oxidative stress is required for the apoptosis induced by TGF-β + PD98059. Cells were incubated in the absence or the presence of TGF-β (2 ng/ml) with or without the MEK inhibitor PD98059 (50 μM), with or without diphenyleneiodonium (DPI, 10 μM) alone or in combination with Glutathione ethyl ester (GEE, 2 mM), as indicated. **A.** Intracellular peroxide content at the indicated times of treatment. Mean ± S.E.M of 3 independent experiments in triplicate. **B.** Caspase-3 activity (16 h of treatment). Mean ± S.E.M. of 3 different experiments. **C.** Transcript levels of *BMF*, *BCL-XL* and *MCL1* after 16 h with the indicated treatments, analyzed by RT-MLPA. Mean ± S.E.M. of 3 different experiments expressed as fold change relative to untreated cells. Student's t test TGF-β+PD98059 treated cells versus TGF-β+PD98059 combined with the antioxidant pre-treatments: *$p < 0.05$; **$p < 0.01$; *** $p < 0.001$.

A careful analysis of the attenuation of cell death in NOX4 targeted knock-down cells (Fig. 28D) revealed that a slight loss in cell viability continued being observed in response to TGF-β + PD98059. For this, we decided to explore whether or not the lysosomal permeabilization, which appeared to be NOX4-independent, might play a role in the TGF-β + PD98059-induced cell death. Among different protease inhibitors, we could only observe a slight, but significant, protecting effect on cell death with the cathepsin B inhibitor Ca-074 (Fig. 31). These results indicate that a cathepsin B-mediated pathway might contribute to TGF-β+PD98059-induced cell death through a parallel pathway that is NOX4-independent.

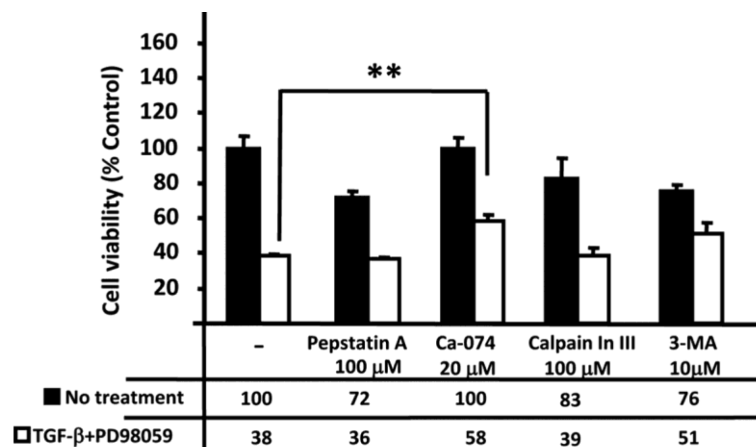


Figure 31. Effect of different protease inhibitors on TGF-β + PD98059-induced apoptosis. Analysis of the cellular viability when cells were pre-treated for 30 min with different protease inhibitors, and then incubated in the absence or the presence of TGF-β (2 ng/ml) and PD98059 (50 μM) for 24 h. Pepstatin A (100 μM, inhibitor of aspartic proteinases such as pepsin, cathepsins D and E), Ca-074 (20 μM, N-(L-3-trans-propylcarbamoyl-oxirane-2-carbonyl)-L-isoleucyl-L-proline, inhibitor of Cathepsin B), Calpain Inhibitor III (100 μM), 3-methyladenine (10 μM, autophagy inhibitor). Data are compared as indicated in the figure, Student's t test t: **$p < 0.01$.

Taking these results into consideration, we suggest that in those HCC cells that show the RAS/MEK/ERK pathway over-activated, TGF-β-induced apoptosis is inhibited due to impairment of NOX4 up-regulation, which is necessary for modulation of the BCL-2 family expression and an efficient mitochondrial-dependent apoptosis.

3. Effects of TGF-β on liver cell phenotype and differentiation state.

3.1. TGF−β induces an EMT process in FaO rat hepatoma cells

When FaO cells were incubated with TGF-β, 50 % of the cells underwent apoptosis but the other 50 % survived. The vast majority of surviving cells acquired a fibroblastic morphology, different from the epithelial FaO cells (Fig. 32A). After 24 h, TGF-β stimulated actin cytoskeleton reorganization, by inducing membrane ruffling, lamellipodia and stress fibers (Figs. 32 B-C). E-cadherin down-regulation and delocalization was observed after 3 days of incubation with TGF-β (Figs. 33A-B). TGF-β induced loss of *E-cadherin (Cdh1)* mRNA levels, which correlated with up-regulation of its transcription repressor *Snail (Snai1)* (Fig. 33C). Interestingly, changes in the phenotype were coincident with the up-regulation of *Tgfb*, the expression of which is silenced in epithelial cells, but it is expressed in mesenchymal cells (Fig. 33C). Finally, TGF-β induced a decrease in the levels of Cytokeratin-18 (Fig. 33D). All these results indicate that TGF-β is inducing an epithelial-mesenchymal transition process in FaO rat hepatoma cells.

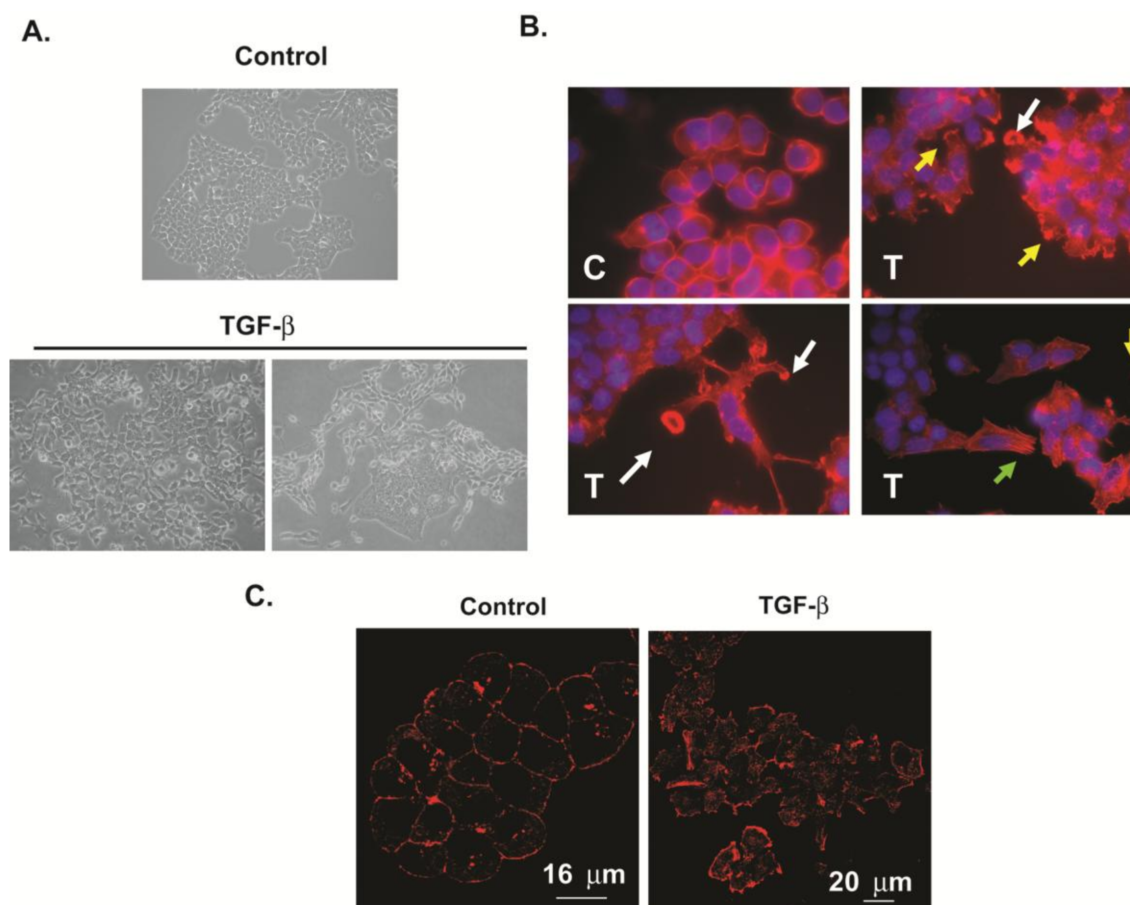


Figure 32. TGF-β induces morphological changes and actin reorganization in FaO rat hepatoma cells. A. Light microscopy photographs of FaO cells incubated for 3 days with or without TGF-β. **B**. Actin filament organization in untreated (C) or TGF-β-treated (24h) FaO cells (T). F-actin filaments were stained with rhodamine-conjugated phalloidin. Blue signal represents the nuclear DNA-staining with DAPI. **C.** The same preparations were analyzed in a Confocal Fluorescence Microscope. In both B and C, a typical epithelial actin distribution is found in control FaO cells, whereas ruffles (yellow arrows), lamellipodia (white arrows) and stress fibers (green arrows) can be observed in FaO-TGF-β treated cells.

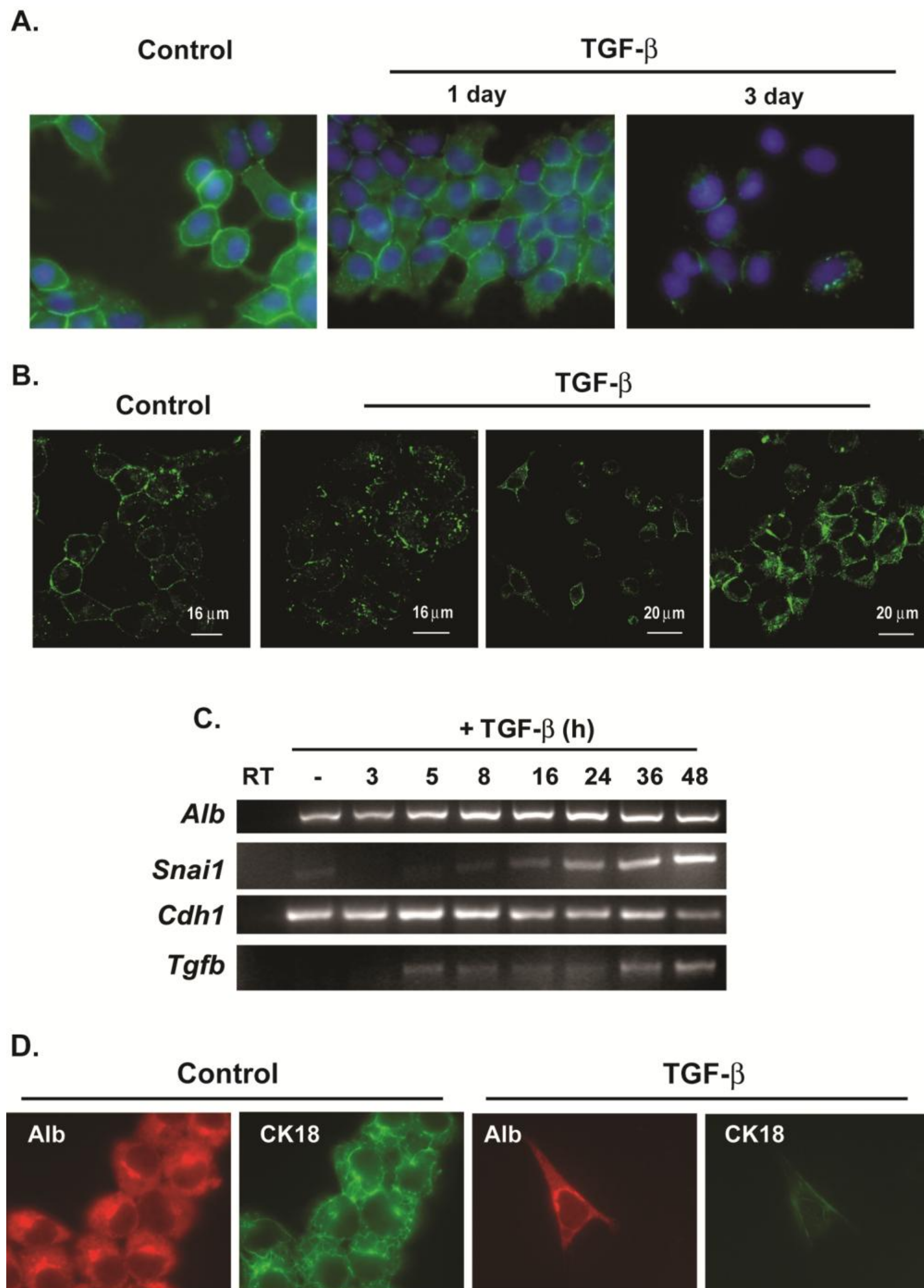


Figure 33. TGF-β induces changes in the expression of Epithelial-Mesenchymal Transition related genes in FaO rat hepatoma cells. A. Immunofluorescence detection of E-cadherin (green) in FaO cells treated with TGF-β for 24 and 72 h. Blue signal represents the nuclear DNA-staining with DAPI. **B**. Confocal images of E-cadherin immunodetection in FaO cells treated with TGF-β (72 h). Loss of pericellular distribution, and lower expression, is observed in TGF-β-treated cells. **C.** Expression of genes related to epithelial-mesenchymal transitions, analyzed by RT-PCR in cells after different times of treatment with TGF-β. *Albumin* transcripts (*Alb*) are shown as control. A representative experiment of four is shown. **D**. Analysis of Albumin (Alb, red) and Cytokeratin-18 (CK18, green) content, by immunofluorescence analysis, in cells cultured with or without TGF-β (24 h). Fibroblastic-like cells in TGF-β treatments lose CK18 expression. A representative experiment from three is shown.

We next wanted to analyze the effects of TGF-β when FaO cells were treated chronically with this cytokine. To isolate the mesenchymal population, FaO cells were submitted to two cycles of TGF-β treatment (2 ng/ml), as indicated in the Fig. 34, in Coon's medium and in the absence of FBS. After removing dead cells, the remaining ones were cultured in the same medium supplemented with 10% FBS (TβT-FaO from TGF-β-treated FaO cells). Cells that survived to TGF-β-induced apoptotic effect showed a mesenchymal-like phenotype, as observed in an inverted microscope (Olympus IX-70). The addition of 0.5 ng/ml TGF-β to the culture medium was enough to maintain the mesenchymal phenotype. However, if the TβT-FaO cells were cultured in the absence of TGF-β, they reverted to an epithelial phenotype in 3-4 weeks (Rev-FaO cells).

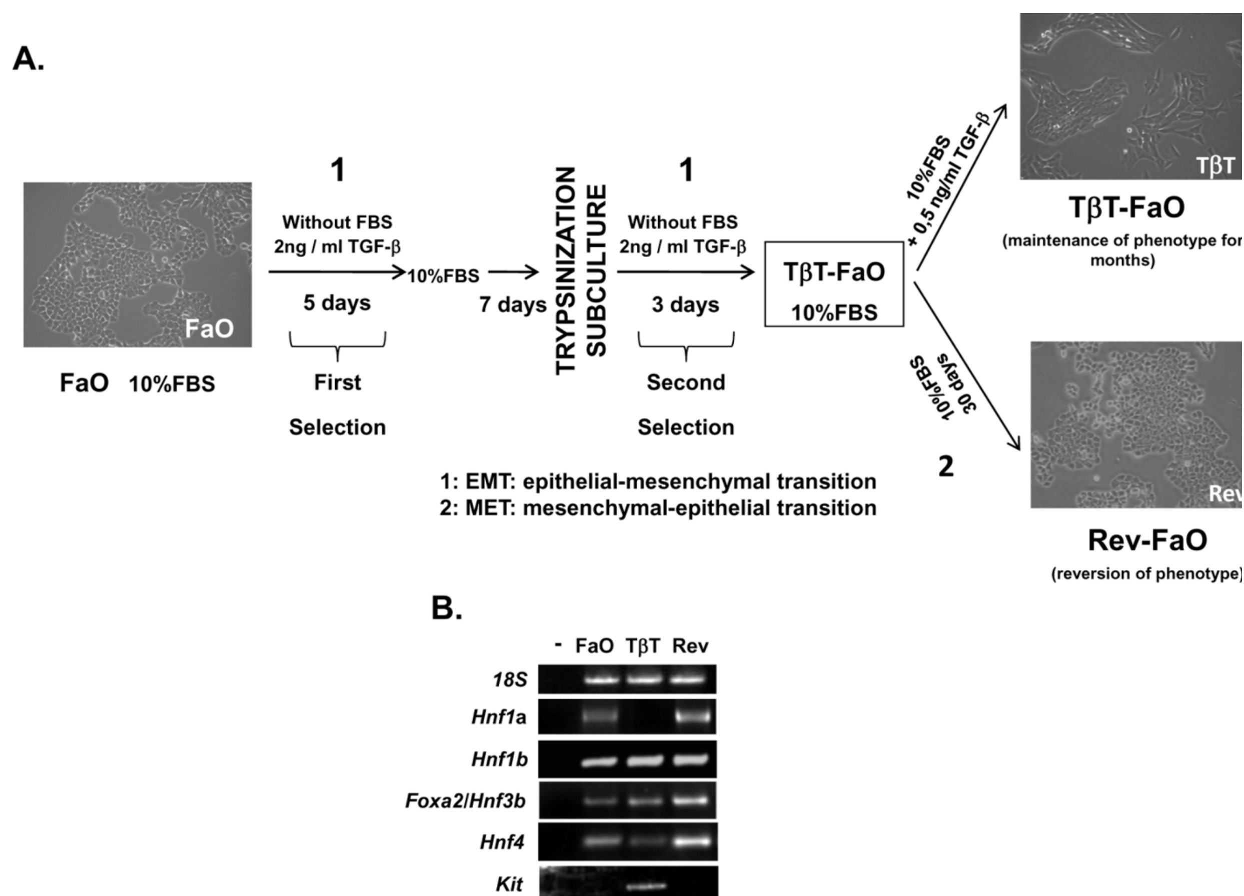


Figure 34. Isolation of FaO rat hepatoma cells that have undergone EMT after treatment with TGF-β. A. Isolation of FaO rat hepatoma cells that have survived to two cycles of TGF-β treatment, as indicated in the Figure (TβT-FaO, from TGF-β-treated FaO cells). Phenotype is maintained when TGF-β (0.5 ng/ml) is present in the culture medium. Removal of TGF-β provokes the reversion to the original epithelial phenotype (Rev-FaO) after 3-4 weeks in culture. Light microscopy photographs display the different morphologies of FaO, TβT-FaO and Rev-FaO cells. **B.** Transcript levels of *Hnf1a*, *Hnf1b*, *Foxa2*/*Hnf3b*, *Hnf4* and *Kit* in FaO, long term TGF-β-treated FaO cells and Rev-FaO were studied to determine their differentiation state. The stated genes, and the *18S* RNA as normalization control, were analyzed by RT-PCR.

TβT-FaO cells not only had a more mesenchymal phenotype, but also presented a de-differentiated state. These cells had lost the expression of the Hepatocyte Nuclear Factors *Hnf4* and *Hnf1a*, characteristic of terminally differentiated liver cells (Fig. 34B) without affecting *Hnf1b* and *Hnf3b*,

which are expressed in earlier stages of liver differentiation. EMT also induced the expression of *c-Kit*, the Stem Cell Factor receptor (Fig. 34B). Interestingly, cells re-differentiated after reversion to an epithelial phenotype.

3.2. TGF−β induces a transdifferentiation process in human fetal hepatocytes and HCC cells

Next, we wanted to know whether human liver cells might undergo EMT, since there were no data in the literature. For this, we used the HFH primary cultures in Dr. Fausto's laboratory. We observed that not all these cells died in response to this cytokine and, as can be observed in Fig. 35, TGF-β induced morphological changes that were coincident with an EMT process characterized by cytoskeleton re-organization and the apparition of stress fibers and lamellipodia. TGF-β induced *Snail (SNAI1)* expression, which was followed by a decrease in *E-cadherin (CDH1)* expression and the up-regulation of mesenchymal markers such as *Vimentin (VIM)* and *N-cadherin (CDH2)* (Fig. 35C). Likewise, we could observe a replacement of intermediate filaments, from the epithelial Cytokeratin-18 (CK18) to the mesenchymal Vimentin analyzed by immunocytochemistry (Fig. 35D). Furthermore, TGF-β diminished the expression of various hepatic markers such as *ALBUMIN* and *HNF4A* at the mRNA level (Fig. 36A).

Human fetal hepatocytes are not fully differentiated cells, showing characteristics of hepatoblast. Indeed, they express several markers that have been described for both hepatoblast/oval cells and hepatic stem cells, such as EpCAM and CK19 (Schmelzer et al. 2006), or specifically for hepatoblasts/oval cells, such as AFP, or for hepatoblasts/oval cells and cholangiocytes, such as CK7 (Libbrecht and Roskams 2002) Here we observed that TGF-β treatment of HFH induced a decrease in the expression of different genes specifically expressed in hepatocytes (Lazaro et al. 2003), such as Albumin (*ALB*), *AFP* or *HNF4A* (Fig. 35A), and immunocytochemistry analysis revealed loss of alpha1-antitrypsin (A1AT) and Connexin 26 (CX26), or the hepatocyte antigen Hepar1, (Fig. 36B). In contrast, TGF-β increased the protein levels of HNF3β, a transcription factor that is expressed at the first stages of the hepatic lineage in non-differentiated cells, and of hepatoblast markers, such as CK7 and 19 (Fig. 36C-D). Interestingly, TGF-β induced an increase in the expression of CD90 (THY1), a stem marker of the hematopoietic lineage also expressed in liver stem cells (Dan et al. 2006) (Fig. 36D). All these results suggest that TGF-β-induced EMT is coincident with a de-differentiation process in human fetal hepatocytes.

Next, we wanted to know whether after chronic treatment with TGF-β, we could isolate this mesenchymal population, as we previously made in fetal rat hepatocytes (Valdes et al. 2002) or in FaO rat hepatoma cells (see above). For this purpose, HFH were treated with 2 ng/ml TGF-β for 72 hours; those cells that had survived to TGF-β-induced apoptosis, and had acquired a mesenchymal phenotype, were later maintained in complete media with 0.5 ng/ml of TGF-β (Fig. 37A: TβT-HFH, from TGF-β-treated HFH). As can be observed in Fig. 37B these cells expressed low levels of some liver specific proteins, such as ALB, A1AT and CX26, and no expression of the hepatocyte antigen Hepar1. TβT-HFH cells also lost the expression of the epithelial CK18 (Fig. 37B). In contrast, they expressed high levels of mesenchymal proteins, such as Vimentin and SMA, and show expression of the stem marker CD90 (THY1) (Fig. 37C)

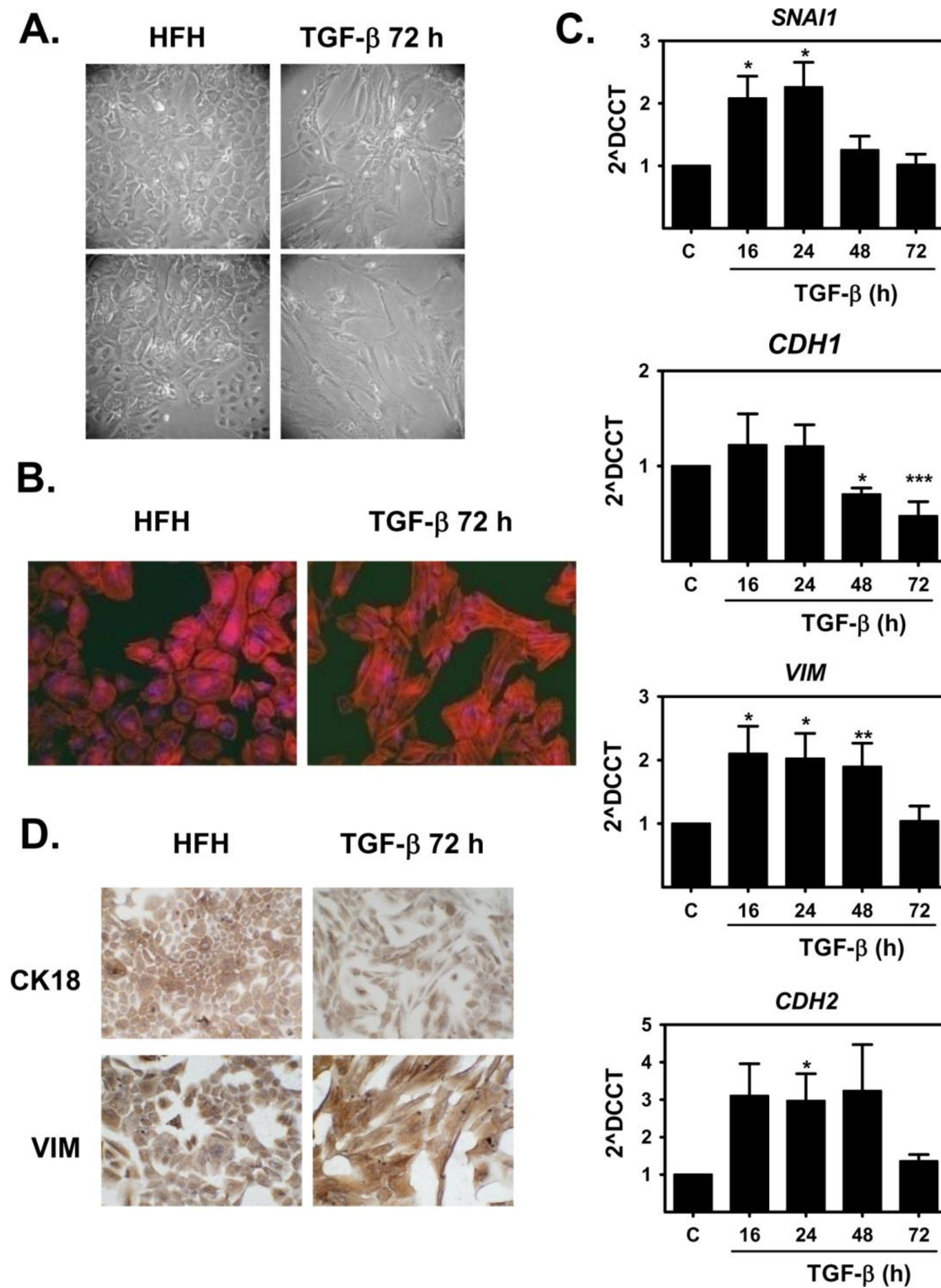


Figure 35. TGF-β induces an epithelial to mesenchymal transition process in human fetal hepatocytes. Human fetal hepatocytes were treated with TGF-β (1 ng/ml) at the indicated times. **A.** Phase-contrast microscopy images. **B.** F-actin filaments stained with Phalloidin-Texas Red, imagined at 40X magnification. **C.** Real-time PCR analysis of the transcript levels of the indicated genes, results expressed as relative to untreated cells and shown as mean ± S.E.M. of 4 different experiments, Student's t test untreated cells versus TGF-β-treated cells: *p <0.05; **p < 0.01; *** p < 0.001. **D.** Immunocytochemistry of CK18 and VIM, imagined at 20X magnification. A, B and D are representative of 4 experiments.

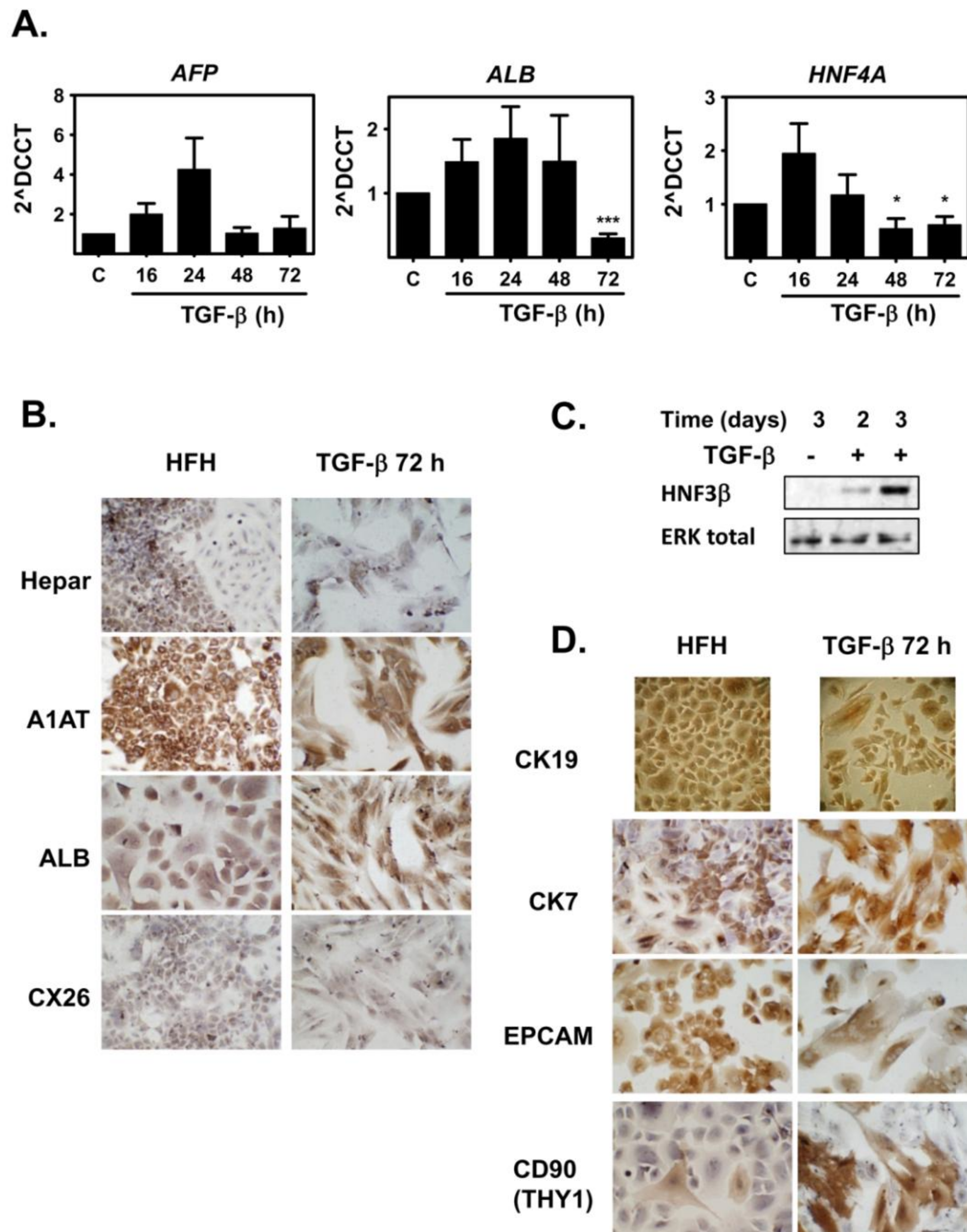


Figure 36. TGF-β induces a de-differentiation process in human fetal hepatocytes. HFH were treated with TGF- β (1 ng/ml) at the indicated times. **A.** Real time PCR analysis of the transcript levels of hepatic differentiation genes, results expressed as relative to untreated cells and shown as mean ± S.E.M. of 4 different experiments Student's t test untreated cells versus TGF- β-treated cells: *p <0.05; *** p < 0.001. **B**. Immunocytochemistry of various hepatic differentiation markers, imagined at 20X magnification. **C.** HNF3β Western Blot, ERKs are used as loading control. **D.** Immunocytochemistry of different hepatic stem cell markers. B, C and D are representative of 4 experiments.

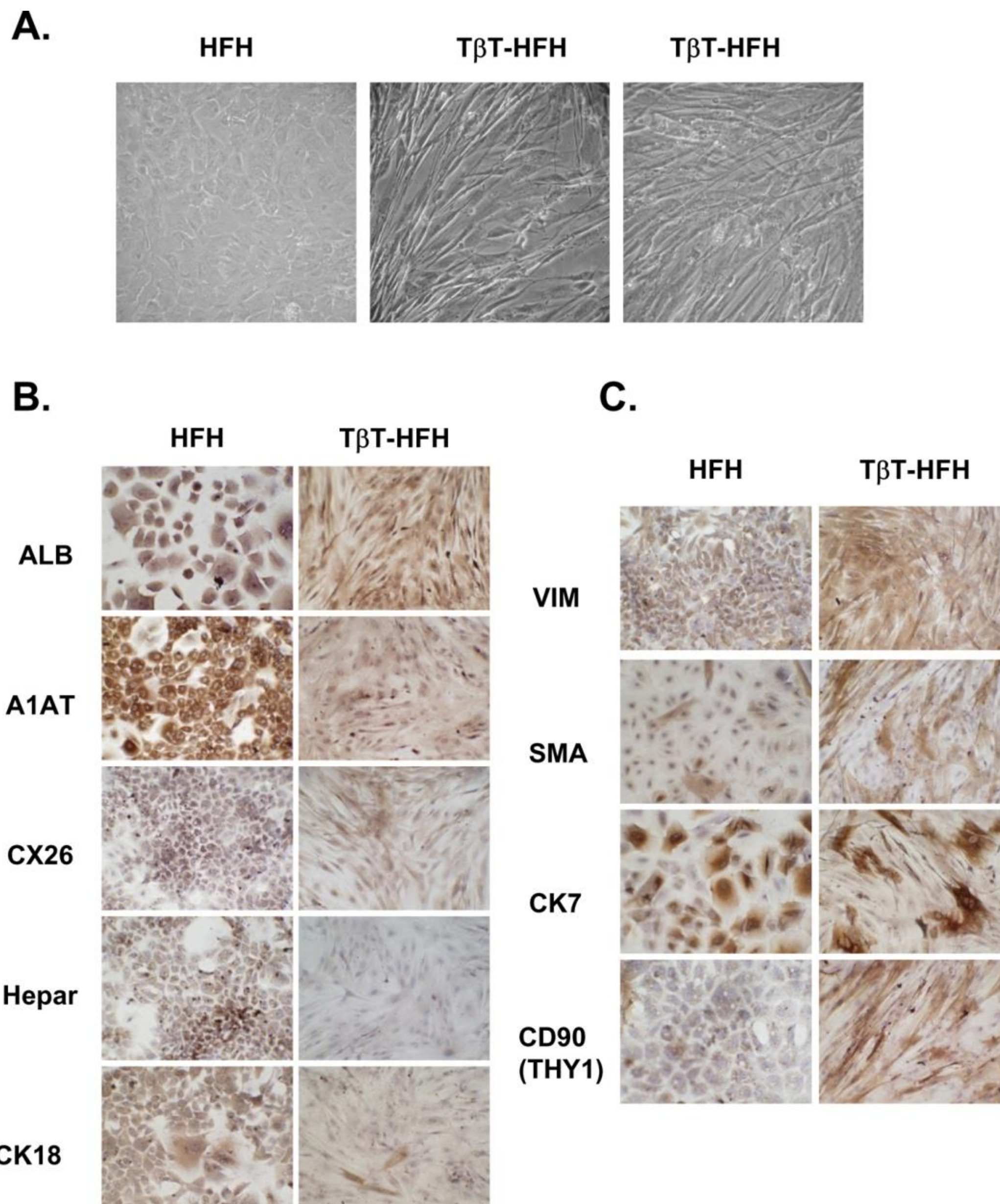


Figure 37. Isolation and characterization of human fetal hepatocytes treated chronically with TGF-β (TβT-HFH). A. Phase-contrast microscopy images. **B.** Immunocytochemistry of various hepatic differentiation markers, imagined at 20X magnification. **C.** Immunocytochemistry of mesenchymal and hepatic stem cells markers. A, B and C are representative of 4 experiments, imagined at 20X magnification.

Next we wanted to know whether TGF-β-induced EMT and de-differentiation might be a reversible process. Therefore, we maintained the TβT-HFH cells with a rich differentiation medium (see Fig. 38 legend), changing it every 2-3 days. This allowed the cells to grow normally, and after 15 days of treatment most cells acquired a more epithelial morphology (Fig. 38A), which was associated with a decrease in the expression of *SNAI1*, and a recovery of the expression of *CDH1*, *ALB*, and *HNF4A* at the transcript level (Fig. 38B). Loss in the protein levels of Vimentin and a recovery of the expression of CK18 and CX26 were observed after maintaining these cells with the differentiation media (Fig. 38C).

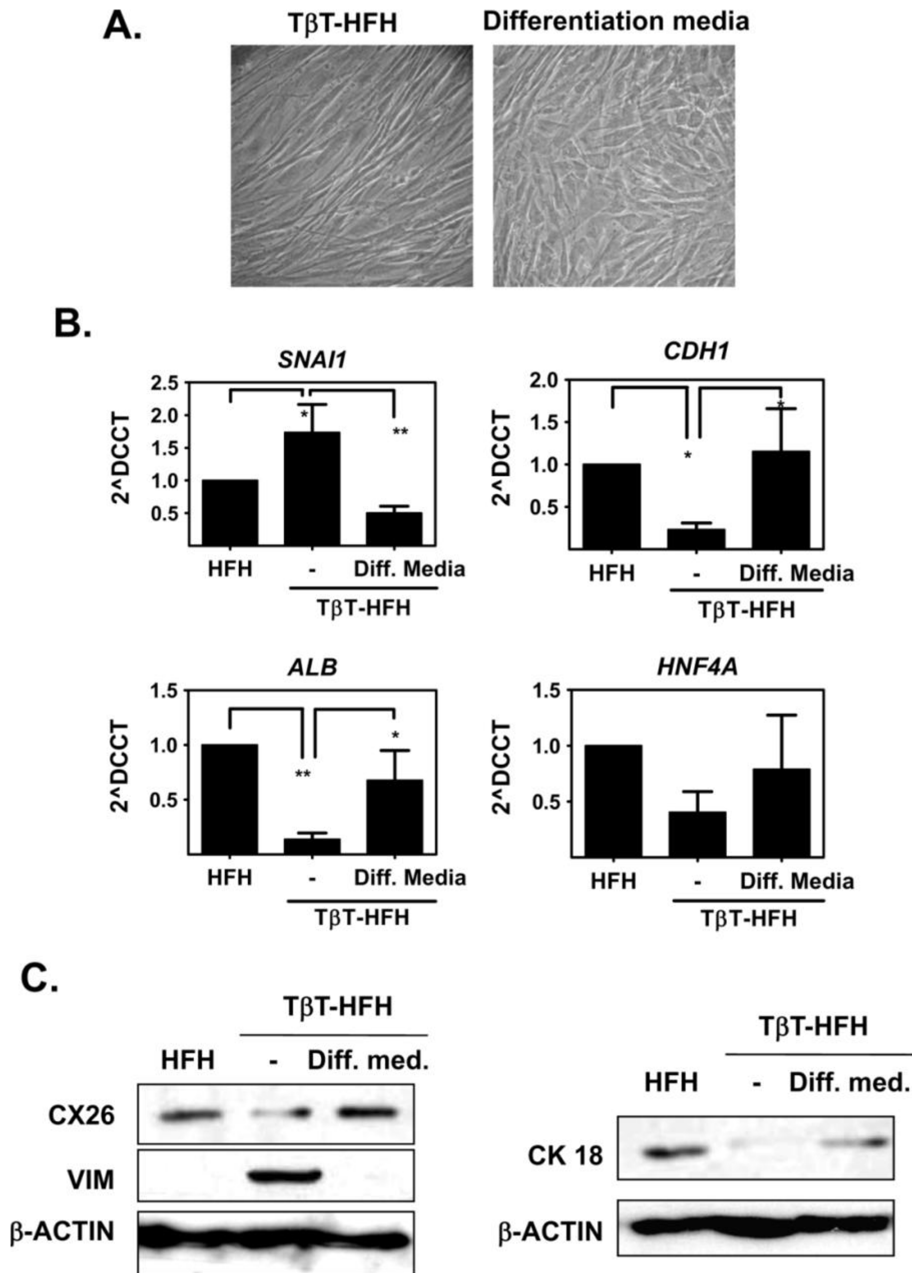


Figure 38. Recovery of hepatic phenotype in HFH-TβT through incubating the cells in a rich differentiation media. HFH-TβT cells were treated during 30 days with a differentiation media (William's E Media, 2 mM Sodium Pyruvate, 14 mM glucose, ITS, Ascorbic Acid, 20 ng/ml HGF, 10 ng/ml Oncostatin, 1 μM Dexamethasone). **A.** Phase-contrast microscopy images. **B.** Real time PCR analysis of the transcript level of hepatic differentiation markers, results expressed relative to untreated cells and shown as mean ± S.E.M. of 4 different experiments. Data are compared as indicated in each figure, Student's t test $^{*}p < 0.05$; $^{**}p < 0.01$. **C.** Analysis of expression at the protein levels of hepatic and mesenchymal markers; β-ACTIN was used as a loading control. A and C are representative experiments of 4.

In summary, TGF-β induces an EMT process in human fetal hepatocytes, coincident with de-differentiation and acquisition of hepatic stem cell markers. This process is reversible, since the culture of TβT-HFH with a differentiation media allows the recovery of the epithelial phenotype and the expression of specific hepatic genes.

Finally, preliminary results obtained in Hep3B cells, showed that TGF-β also induced *SNAI1* expression at the mRNA level after only one hour of treatment, which was followed by a down-regulation of *CDH1* expression at later times. TGF-β also increased the expression of mesenchymal markers such as *VIM* and *CDH2* (Fig. 39A). These changes in mRNA expression were accompanied by changes in cell morphology and a reorganization of actin cytoskeleton, with the apparition of ruffles and lamellipodia (Fig. 39B). Moreover, by immunocytochemistry we could observe a decrease in the protein levels of E-cadherin, as well as its delocalization from the membrane to intracellular compartments, a decrease in the number of cells that showed CK18 filaments and an increase in those showing Vimentin (Fig. 39C). It is worthy to note that Hep3B cells, as many other HCC cells, do not show a pure epithelial phenotype and cells express Vimentin, although at lower levels, even before TGF-β treatment.

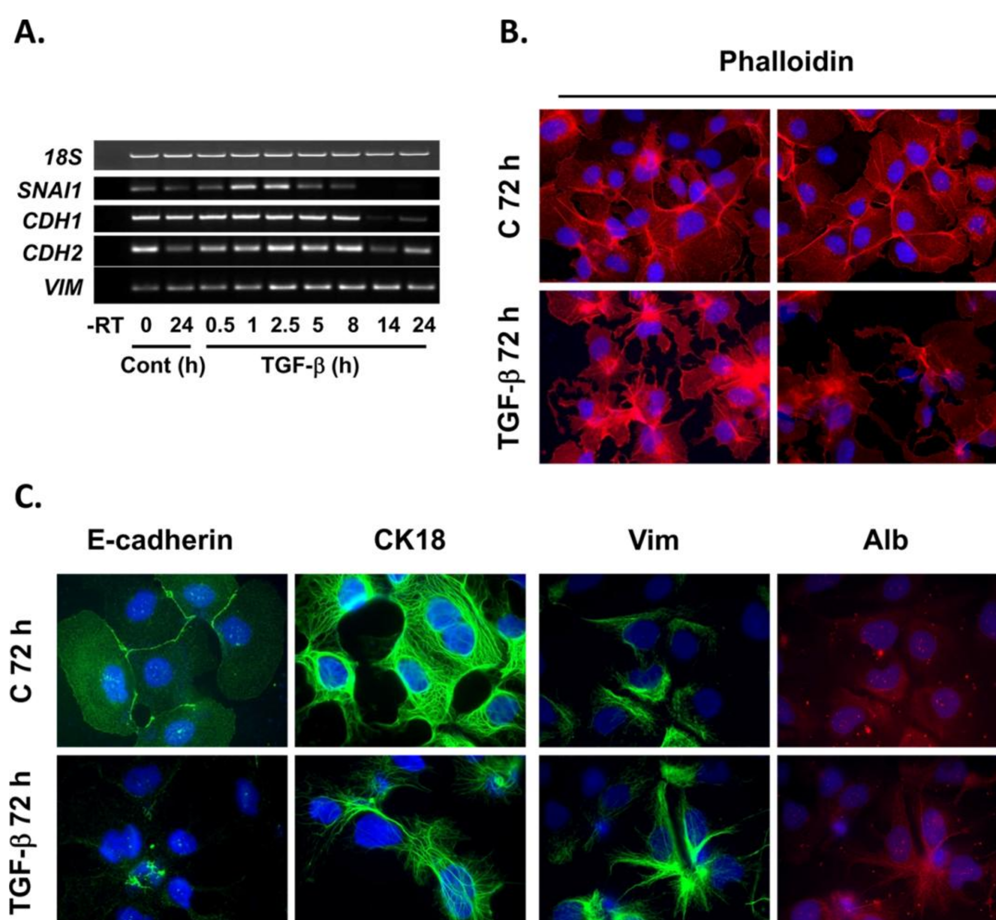


Figure 39. TGF-β induces an Epithelial-Mesenchymal Transition process in Hep3B cells. **A.** Expression of genes related to epithelial-mesenchymal transitions, analyzed by RT-PCR in cells after different times of treatment with TGF-β. *18S* transcripts are shown as control. **B.** F-actin filaments were stained with rhodamine-conjugated phalloidin. Blue signal represents the nuclear DNA-staining with DAPI, imagined at 60X magnification. **C.** Immunofluorescence detection of E-Cadherin, Vimentin, CK18 and Alb in Hep3B cells treated with TGF-β for 72 h, imagined at 100X magnification. In each case a representative experiment of 3 is shown.

The EMT process in Hep3B cells was also accompanied by cell de-differentiation, since TGF-β induced a decrease in the expression of hepatic markers such as *ALB* and *AFP*, and up-regulation of stem cell markers, such as *THY*, *KIT* and *EPCAM* (Fig. 40).

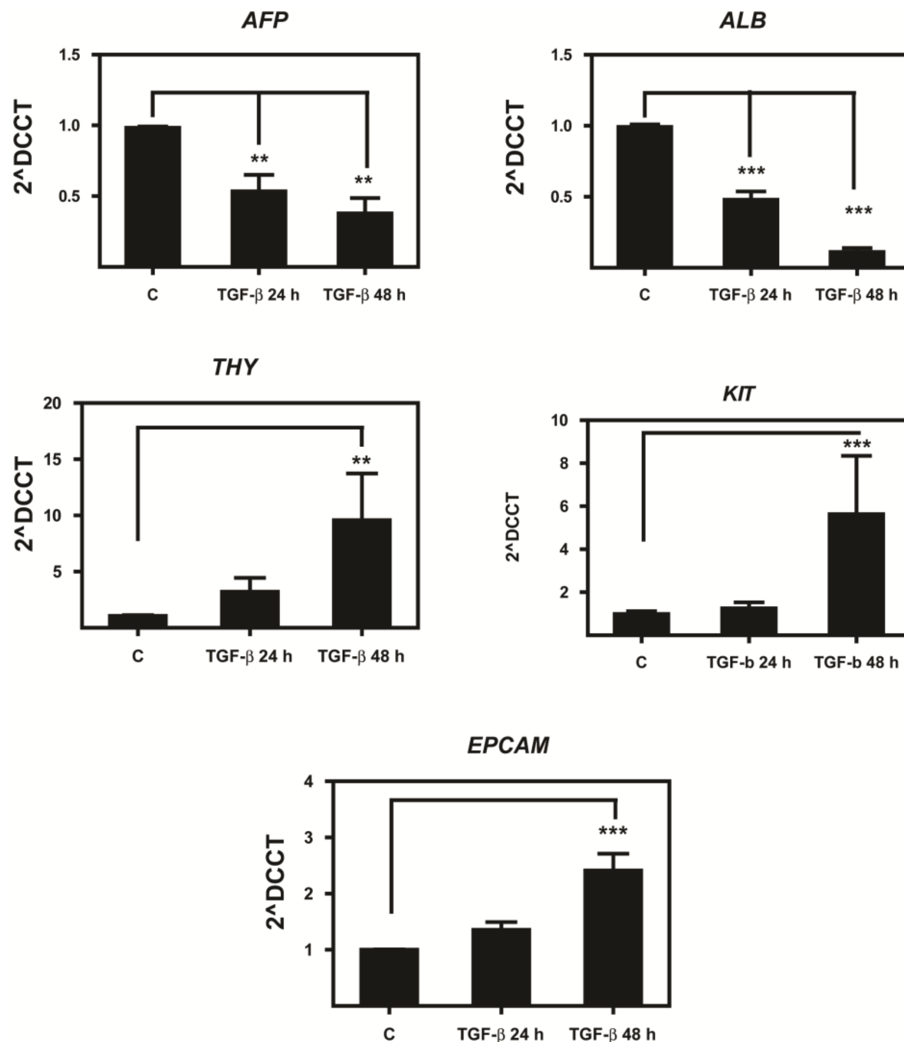


Figure 40. TGF-β induces a de-differentiation process in Hep3B cells. Real time PCR analysis of the transcript levels of hepatic differentiation and stem cell markers in Hep3B cells treated with 2 ng/ml TGF-β during 24 and 48 hours, results expressed as relative to untreated cells and shown as mean ± S.E.M. of 2 different experiments. Student's t test as indicated in each figure: *p <0.05; **p < 0.01; *** p < 0.001.

In conclusion, TGF-β induces EMT both in non-transformed human fetal hepatocytes and in human liver tumor cells, a process that is coincident with cell de-differentiation. After chronic treatment with TGF-β, the mesenchymal phenotype can be reversed back to an epithelial one by removing TGF-β from the media and incubating the cells in the presence of differentiation factors.

VIII. DISCUSSION

The transforming growth factor beta (TGF-β) is an important growth inhibitor and apoptosis inducer in different cell types (Massague 2008). Although there is no doubt about its role as tumor suppressor, it has been clearly demonstrated that it also contributes to tumor progression and metastasis through the induction of other effects, such as EMT processes, which increase cell migration and invasion (Valcourt et al. 2005; Zavadil and Bottinger 2005). TGF-β also plays contradictory roles in liver development and carcinogenesis. On the one hand, TGF-β inhibits proliferation, suppresses transformation and induces apoptosis during liver carcinogenesis, and disruption of TGF-β signaling can deregulate apoptosis in HCC (Bissell et al. 2001; Siegel and Massague 2003). On the other hand, TGF-β plays a major role as a tumor promoter by inducing EMT processes which increase hepatocyte ability to migrate (Gotzmann et al. 2002). Indeed, its activation has been associated with the progression of hepatocarcinogenesis. Accordingly, the expression of TGF-β itself is often increased in HCC (Dong et al. 2008) and the constitutive expression of mature TGF-β in the liver accelerates hepatocarcinogenesis in transgenic mice (Schnur et al. 1999). During carcinogenesis, TGF-β tumor suppressor responses are lost but its tumor progression effects are maintained (Wakefield and Roberts 2002; Seoane 2006). Indeed, the escape from the antiproliferative and pro-apoptotic actions of TGF-β might be a prerequisite for hepatocarcinoma progression (Yang et al. 2006). Furthermore, considering the ability of TGF-β to induce epithelial-mesenchymal transitions in liver cells (Gotzmann et al. 2002; Valdes et al. 2002), the possible response of hepatoma cells to TGF-β inducing a more fibroblastic/migratory phenotype would convert this cytokine in a fibrotic and invasion-promoting factor. This dual role of TGF-β in human liver tumor progression has been evidenced in a recent work by Dr. Thorgeisson's group, who studied a cohort of tumor tissues from HCC patients and defined two different TGF-β gene signatures, which correlated with differences in malignancy and invasive potential of the tumors. To do so, first they studied the genes modulated by TGF-β in mouse hepatocytes, dividing them into two sets of genes: 1) TGF-β-induced early response genes, modulated during the first two hours of treatment, these genes being related to growth inhibition and apoptosis control; 2) TGF-β-induced late response genes, modulated between 4-24 hours after TGF-β treatment, which were involved in migration and anti-apoptotic actions. They then studied the gene expression profile of different HCC tumors and human HCC cell lines, and classified them in accordance to an early or late TGF-β gene signature. They observed that those tumors which had a late TGF-β signature showed poorer prognosis, with increased recurrence and invasivity (Coulouarn et al. 2008). Thus, the elucidation of the signaling induced by TGF-β in human hepatocytes and liver tumor cells, and their possible crosstalks with other intracellular signals that might impair its suppressor effects, would have relevance in the future design of therapeutic tools to balance the responses of TGF-β in favour of liver tumor suppression.

1. TGF-β induces both pro- and anti- apoptotic signals in liver tumor cells. Role of Nox4 and EGFR.

Our group has previously described that TGF-β induces apoptosis in rat adult and fetal hepatocytes (Sanchez et al. 1996; Caja et al. 2007). Here we have observed that TGF-β is able to induce growth inhibition and apoptosis in human fetal hepatocytes (HFH) and in some liver tumor cells, such as the FaO rat hepatoma cells, or the Hep3B and PLC/PRF/5 human hepatocarcinoma cells. However, some liver tumor cells are refractory to its suppressor effects, such as the HepG2 and SK-Hep1 cell lines (Figs. 1, 5, 7 and 8). The different responses obtained in the various cell lines used after TGF-β

treatment allowed us to compare its effects in each model in order to find what makes a cell sensitive to TGF-β-induced apoptosis, and what confers resistance to this cytokine's suppressor effects.

Several reports have implicated members of the BCL-2 family in mediating TGF-β-induced apoptosis (Chatzaki et al. 2003; Ramjaun et al. 2007; Gingery et al. 2008; Spender et al. 2009). In the two TGF-β-sensitive cell models that we studied, HFH and Hep3B, TGF-β significantly induces the expression of different pro-apoptotic members of the BCL-2 family, mainly BMF and BIM, (Figs. 6B and 9B). In both cell models, TGF-β also up-regulates the expression of anti-apoptotic members of the BCL-2 and IAP family, in particular, BCL-XL, MCL1, XIAP (Figs. 6B and 9B). BMF would act as sensitizer and BIM as an activator in order to promote BAX and BAK activation, while sequestering BCL-XL and MCL1 proteins (Letai et al. 2002; Willis and Adams 2005; Merino et al. 2009). Therefore as long as TGF-β induces the expression of the pro-apoptotic proteins, it will induce apoptosis. However, this process might be attenuated by the increased expression of anti-apoptotic proteins after TGF-β treatment, final TGF-β-induced apoptosis being dependent on the ratio of pro- versus anti-apoptotic proteins.

TGF-β-induced apoptosis requires ROS production in hepatocytes (Sanchez et al. 1996; Franklin et al. 2003; Black et al. 2007), which precedes loss of mitochondrial transmembrane potential (Herrera et al. 2001a) and it depends on Smad3 expression (Black et al. 2007). We knew that TGF-β increases ROS intracellular levels in rat fetal hepatocytes through a NAPDH oxidase like system and decreases the expression levels of different antioxidant proteins (Herrera et al. 2004), creating an oxidative stress state that would lead to apoptosis (Herrera et al. 2001a). Here we show that TGF-β also induces the expression of NOX4 in human fetal hepatocytes (Fig. 6A), which is coincident with an increase in ROS production in response to TGF-β. We show here that not all the human HCC cell lines analyzed are sensitive to TGF-β. Interestingly, TGF-β-induced apoptosis correlates with its ability to up-regulate NOX4 expression. Indeed, apoptosis (Fig. 8) is neither induced in HepG2 cells by TGF-β, nor is the expression of NOX4 increased (Fig. 10A), whereas Hep3B cells respond to TGF-β up-regulating NOX4, which is coincident with an increase in the levels of intracellular ROS (Fig. 10B) and is required for a mitochondrial-dependent apoptosis (Figs. 8-11). These results indicate that an efficient apoptotic response to TGF-β in human hepatocellular carcinoma cells might depend on their capacity to respond to TGF-β in terms of NOX4 up-regulation.

The fact that ROS act as intermediate signals in different physiological processes in cells has grown in acceptance since the NOX family was discovered. These enzymes are expressed in numerous cell types and play essential roles including gene expression, cell death, differentiation, proliferation and migration (Bedard and Krause 2007; Brown and Griendling 2009). Various articles have reported the involvement of NOX proteins in cell survival and apoptosis. NADPH oxidase proteins have been described to be involved in CD95-dependent apoptosis in hepatocytes (Reinehr et al. 2005), and TNF-α has been shown to activate NOX1 in fibroblasts to promote necrosis (Kim et al. 2007). NOX4 has been proposed to have opposite roles in different cell systems: firstly, in response to different stimuli, NOX4 is involved in apoptosis in macrophages (Palozza et al. 2007), leukemia cells (McKallip et al. 2006), endothelial cells (Basuroy et al. 2009) and hepatoma cells (Song et al. 2009); secondly, NOX4 is also involved in the induction of proliferation and survival signals in human aortic smooth muscle cells (Sturrock et al. 2006), pulmonary artery adventitial fibroblasts (Li et al. 2008), pancreatic cells (Vaquero et al. 2004; Edderkaoui et al. 2005), gliomas cells (Shono et al. 2008), as well as in leukemia cells when induced by Bcr-Abl (Naughton et al. 2009). TGF-β-induced NOX4 expression has been described to be involved in cardiac fibroblast differentiation (Cucoranu et al. 2005), and in

the proliferation of human aortic smooth muscle cells (Sturrock et al. 2006). Results shown in this manuscript propose, for the first time, the requirement of NOX4 in TGF-β-induced apoptosis in HCC cells. NOX4 is different to the other members of the family, since its activity is only dependent on its protein levels, although it can be enhanced when associated to p22-PHOX (Ambasta et al. 2004; Serrander et al. 2007). Furthermore, in contrast to other members of the family that are mainly located in the cell membrane, NOX4 localizes in focal adhesions (Hilenski et al. 2004), the nucleus (Kuroda et al. 2005), the mitochondria (Block et al. 2009) and the endoplasmic reticulum (Ambasta et al. 2004). Therefore, NOX4 might regulate protein oxidation in the area surrounding its localization, generating signals, including regulation of activity of kinases, phosphatases and transcription factors. The final balance among those actions might be dependent on the cell phenotype and/or the cell context.

TGF-β not only induces pro-apoptotic signals, but also induces survival signals. As can be observed, the pro-apoptotic effects of TGF-β are not striking, and there are always around 50 % of cells that survive to the cytotoxic effects of this cytokine in FaO cells, being this percentage even higher in HFH, Hep3B and PLC/PRF/5 cells (Figs. 1, 5 and 7). Other groups as well as ours have already described that TGF-β promotes survival pathways through the activation of EGFR in fetal rat hepatocytes (Murillo et al. 2005), endothelial cells (Vinals and Pouyssegur 2001) or breast cancer cells (Wang et al. 2008b). Our work shows that TGF-β activates AKT, ERK and SRC in both FaO and Hep3B cells (Figs. 2 and 12), which is mediated by the EGFR pathway (Fig. 3), a process that is coincident with up-regulation of different EGFR ligands expression (Figs. 2 and 12) and requires TACE activity (Fig. 3).

Human hepatocarcinoma (HCC) is one of the most common cancers, and its prevalence is arising in the West. Surgical resection and liver transplantation are currently the best options to treat liver cancer. However, recurrence or metastasis is common in patients who have had a resection, and survival rate is 30 % to 40 % at 5 years post-operatively (Aravalli et al. 2008). Therefore, more studies are needed to understand the molecular events leading to liver carcinogenesis and how they induce chemo-resistance. A relevant number of molecular mechanisms altered in HCC initiation and progression are compromising the balance between survival and apoptosis in the pre-neoplastic hepatocytes. Some physiological pro-apoptotic molecules are down-regulated or inactivated in HCC, but the balance between death and survival is mainly broken due to over-activation of anti-apoptotic signals (Mott and Gores 2007; Fabregat 2009). Among the de-regulated pathways that causes the cell to replicate at a higher rate and/or results in the cell avoiding apoptosis, is the EGFR pathway (Berasain et al. 2007; Fabregat et al. 2007). For instance, over-expression of TGF-α, Amphiregulin and HB-EGF has been observed in liver cirrhosis and hepatocellular carcinoma, and these factors are believed to contribute to the EGFR activation during hepatocarcinogenesis (Inui et al. 1994; Chung et al. 2000; Berasain et al. 2005). Furthermore, dysregulation of the EGFR system in human HCC tissues includes the over-expression of EGFR and ERBB3 (Berasain et al. 2009).

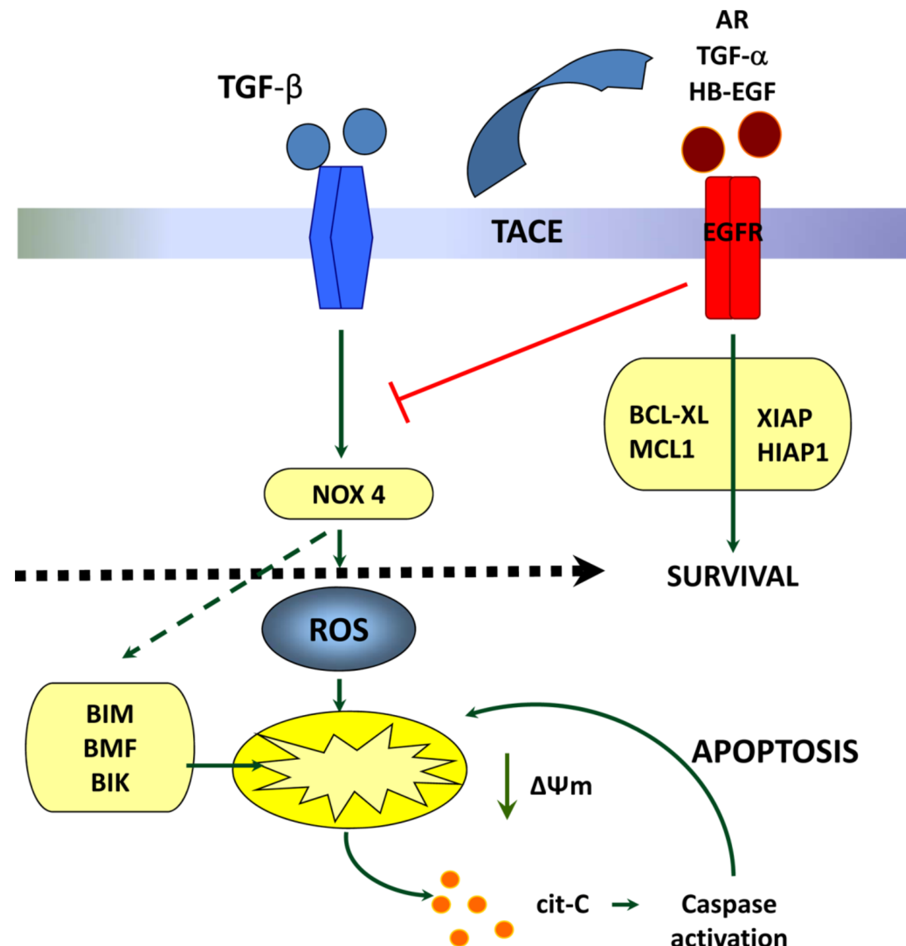
Results presented here indicate that liver tumor cells show basal activation of the EGFR pathway when cultured in the absence of serum. Indeed: 1) they express EGFR ligands, such as Amphiregulin, TGF-α and HB-EGF (Figs. 2 and 12); 2) they show a basal activation of AKT, SRC and ERK1/2 (Figs. 2 and 12); 3) EGFR localizes in the cytosol and/or intracellular vesicles (Fig. 2); and 4) they show basal phosphorylation of the EGFR protein (Fig. 13). EGFR activation mediates cell proliferation, since EGFR knockdown attenuates autocrine growth in Hep3B, HepG2 and SK-Hep1 cells (Figs. 13 and 18). Similar results were obtained with Gefitinib in HFH, Hep3B, PLC/PRF/5, HepG2 and SK-Hep1 cells (Figs. 5, 17 and 19). Moreover, in Hep3B cells, EGFR silencing decreases

phospho-AKT levels and activates caspase-3 (Fig. 13), coincident with a decrease in the expression of the anti-apoptotic gene HIAP1 (Fig. 15), which correlates with an enhancement in the percentage of cells with activated BAK (Fig. 15). Intriguingly, it had no effect on ERKs phosphorylation (Fig. 13), which might be mediated by other growth factors in an autocrine manner.

It is well known that the EGF pathway impairs TGF-β-induced apoptosis in hepatocytes and hepatoma cells (Shima et al. 1999; Herrera et al. 2001a; Herrera et al. 2002). Our results here indicate that, besides inducing a cytotoxic effect per se, the EGFR blockage potentiates TGF-β-induced apoptosis, specifically only in those cells which were sensitive to its suppressor effect: FaO rat hepatoma cells, HFH, PLC/PRF/5 and Hep3B (Figs. 1 and 14-17). However, it had no effect on the HCC cell lines resistant to this cytokine (HepG2 and SK-Hep1, Figs. 18-19). EGFR targeted knock-down or the use of AG1478 or Gefitinib in Hep3B cells increases TGF-β-induced cell death (Figs. 14-17). In the HCC cells that showed an enhancement in the suppressor effects of TGF-β when the EGFR was targeted knock-down, this effect is always coincident with increased up-regulation of NOX4. This event is triggered with each of the methods used for EGFR inhibition (Figs. 4, 6 and 14). These results confirm previous data obtained by our group in rat fetal hepatocytes, where the use of AG1478 increased NOX4 expression levels after treatment with TGF-β (Carmona-Cuenca et al. 2008). Furthermore, we have recently shown that NOX4 silencing impairs the enhancing effects of AG1478 on the pro-apoptotic activity of TGF-β in FaO rat hepatoma cells (Sancho et al. 2009), emphasizing the role of NOX4 in TGF-β-induced cell death.

Here we describe the mechanism by which the blockage of EGFR potentiates TGF-β mediated apoptosis, i.e., by enhancing or repressing the effects of TGF-β on the expression of both BCL-2 and IAP family members. Different responses can be observed, depending on the cell type and the mechanism used for EGFR inhibition. Indeed, in Hep3B cells, EGFR silencing significantly increases the TGF-β-induced expression of *BIK*, *BMF* and *BIM;* and enhances *HIAP1* down-regulation (Fig. 15). In the case of HFH, Gefitinib does not enhance the effects of TGF-β on the expression of pro-apoptotic members of the BCL-2 family, but it counteracts TGF-β-induced expression of anti-apoptotic proteins of both BCL-2 and IAP families (Fig. 6); in this same line of evidence, the use of AG1478 in Hep3B cells does not enhance the effects of TGF-β on the expression of the pro-apoptotic members of BCL-2 family, but it decreases the expression of the anti-apoptotic proteins MCL1 and BCL-XL when used alone or in combination with TGF-β, avoiding their increased expression induced by TGF-β (Figs. 9 and 15). The results obtained with AG1478 in Hep3B are consistent with previous results from our group in which we show that AG1478 alone decreased the expression of both MCL1 and BCL-XL in FaO rat hepatoma cells (Ortiz et al. 2008), an effect that is attenuated when cells are pretreated with a permeable form of GSH (GEE), indicating that oxidative stress is involved in AG1478 regulation of the expression of these anti-apoptotic proteins. Other groups have shown that the use of EGFR inhibitors promote the intrinsic pathway of apoptosis by modulating BCL-2 family gene expression in oral squamous cell carcinoma cells (Takaoka et al. 2007) and in non-small-cell lung cancer cells (Ling et al. 2008). In summary, we propose here that TGF-β activation of EGFR counteracts its pro-apoptotic effects at two different levels: on the one hand, EGFR activation attenuates the up-regulation of NOX4 and the increase in the levels of the pro-apoptotic genes BMF, BIM or BIK; on the other hand, EGFR enhances the expression of some anti-apoptotic proteins of BCL-2 and IAP families (see scheme 1). It has been shown that HBV infection promotes up-regulation of HIAP1 and HIAP2 (Lu et al. 2005), and, in general, the IAP family gene expression is altered in HCC (Notarbartolo et al. 2004; Augello et al. 2009). Additionally, the expression of anti-apoptotic members of the BCL-2 family is also over-expressed in HCC (Takehara et al. 2001;

Fabregat 2009). Thus, the use of therapeutic drugs which directly or indirectly decrease the expression of these anti-apoptotic proteins will enhance TGF-β anti-tumorigenic effects.



Scheme 1. TGF-β induces pro- and anti-apoptotic signals in liver cells

Recent studies in the genetic profiling of human HCC have indicated that some mouse models mimic poor-prognosis human HCC (Lee et al. 2004). Interestingly, the relative similarity of Myc/TGF-α transgenic mice to the human poor survival group in HCC has established a role for EGFR in the prognosis of human HCC (Lee and Thorgeirsson 2005), which indicates the interest in future studies examining expression level, constitutive phosphorylation and mutations of EGFR. Results presented in this work indicate that a parallel analysis of the expression of EGFR ligands, as well as TACE/ADAM17 expression and activity, should complement information about the EGFR pathway in HCC patients. This information might determine the therapeutic potential of EGFR tyrosine kinase inhibitors in a subclass of HCC patients. HCC is often diagnosed at an advanced stage, when it is not amenable to curative therapies. Advances in cancer biology suggests that a limited number of pathways are responsible for initiating and maintaining dysregulated cell proliferation in HCC (Roberts and Gores 2005). Furthermore, accumulating evidence suggests that a defective apoptotic process might play an important role in the promotion stage of HCC (Zender et al. 2006; Fabregat et al. 2007). We can conclude that TGF-β induces both pro- and anti-apoptotic signals in FaO, HFH, Hep3B and PLC/PRF/5 similarly to rat fetal hepatocytes (Valdes et al. 2004; Murillo et al. 2005) through the EGFR. In HCC, the inhibition of EGFR would be a useful therapeutic target since it

would result in the inhibition of autocrine proliferation of hepatoma cells, and it would restore TGF-β pro-apoptotic signaling, as this cytokine's levels are elevated in HCC (Song et al. 2002; Dong et al. 2008). However, this effect would only be expected in those cells that do not show alterations in other survival pathways downstream from the EGFR.

2. Overactivation of MEK/ERK pathway counteracts TGF-β-induced apoptosis in human hepatocarcinoma cells.

Alteration of RAS pathway is frequently observed in HCC, mainly due to RAS mutations and/or genetic or epigenetic silencing of inhibitors of the RAS network, RASSF1A and NORE1A (Calvisi et al. 2006; Calvisi et al. 2008), or activated through the Hepatitis B virus protein X (Tarn et al. 2001). It was recently reported that in HCC up-regulation of H-RAS >3-fold was associated with early recurrence (Newell et al. 2009). RAS activation leads to survival signaling that is transduced from RAS through the serine/threonine kinase RAF, which in turn phosphorylates and activates MAPK kinases MEK1/2 and thereby the p42/44 MAPK (ERK1/2). RAS pathway alteration might confer proliferative and anti-apoptotic properties to neoplastic liver cells (Calvisi et al. 2008). Indeed, several advances in recent years have focused increasing attention on the role of the RAF/MEK/ ERK1/2 pathway in promoting cell survival (Balmanno and Cook 2009).

The present work shows, that once the MEK/ERK pathway is inhibited by specific drugs (PD98059, U0126 or PD325901) (Fig. 20 and 23) or by ERK1/2 silencing (Fig. 24), the TGF-β apoptotic response is restored in HepG2 resistant cells as measured by cell viability assays, caspase-3 activation and percentage of hypodiploid cells. Moreover, MEK inhibition potentiates TGF-β effects in the sensitive cell lines Hep3B and PLC/PRF/5 (Fig. 21). Indeed, over-activation of the RAS/MEK/ERK pathway in liver tumor cells might confer them resistance to the apoptotic effects of TGF-β which is highly expressed during liver tumor progression (Luo et al. 2006). Disruption of the TGF-β suppressor arm occurs in advanced stages of HCC (Kawate et al. 1999; Yakicier et al. 1999; Coulouarn et al. 2008). Although some perturbations at receptor or SMAD levels have been proposed (Yang et al. 2006; Tang et al. 2008), molecular mechanisms are not completely understood. In the case of the HepG2 cell line, which shows alterations in the RAS pathway (Hsu et al. 1993), we observed that even though these cells do not respond to TGF-β-induced cytotoxic effects, they still induce SMAD2 phosphorylation after treatment with TGF-β (Fig. 20), and express all R-SMADs, co-SMAD, I-SMADs and TGF-β receptors I and II (Fig. 22). Therefore, it seems that an alteration of the TGF-β canonical cascade is not responsible for the resistance shown in these cells.

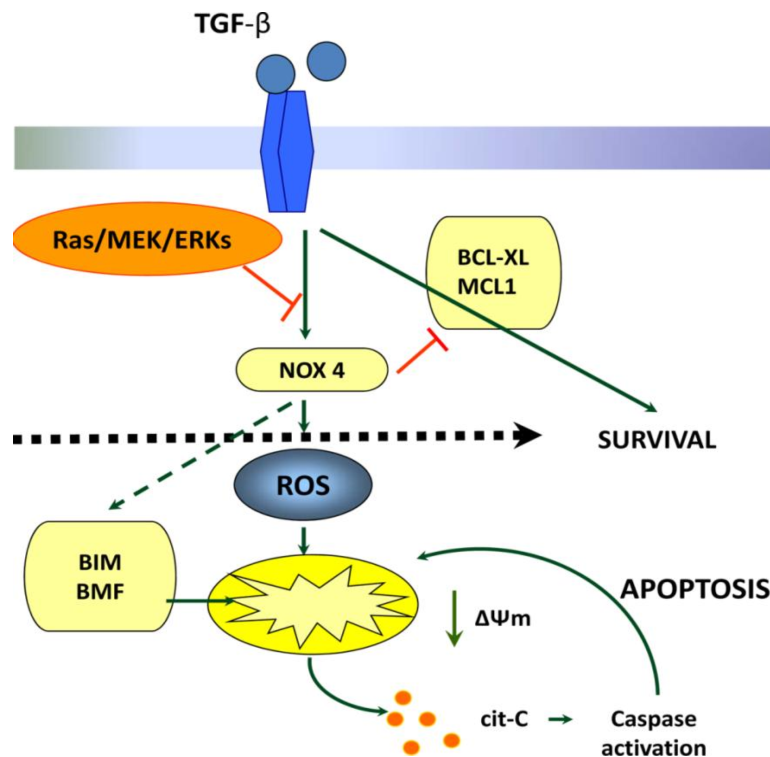
We propose that the crosstalk between the ERKs and TGF-β signaling might be located on a key modulator of ROS production and intracellular oxidative stress: the NADPH oxidase NOX4. Up-regulation of NOX4, ROS production and oxidative stress do not occur in response to TGF-β except when the MEK/ERK pathway is inhibited in HepG2 cells (Fig. 26), which constitutively show phosphorylation of the ERKs. NOX4 regulation by TGF-β in fetal rat hepatocytes occurs at the transcriptional level and it is impaired by the PI3K and MEK/ERK pathways (Carmona-Cuenca et al. 2008). Our results confirm that a similar response must exist in humans and suggest that over-activation of survival signals in HCC cells might be counteracting TGF-β-induced cell death through attenuating NOX4 up-regulation. Interestingly, ERK activation also contributes to the up-regulation of antioxidant genes by TGF-β, such as HO-1 (Fig.27). Here we describe for the first time that the

expression of NOX4 is required for an efficient mitochondrial-dependent apoptosis by TGF-β. NOX4 is necessary, at least, for the up-regulation of two pro-apoptotic BH3-only genes: BMF and BIM (Figs, 25 and 29). It was previously reported that TGF-β regulates the expression, at the transcriptional level, of BMF through a ROS-dependent mechanism (Ramjaun et al. 2007) . Here we suggest that the ROS producing system involved in this process is NOX4. In the case of BIM we find that regulation occurs at the post-transcriptional level. Interestingly, it has been recently suggested that TGF-β regulates BIM by a post-translational mechanism involving the SMAD3-dependent expression of the MAPK phosphatase MKP2 (Ramesh et al. 2008). Our results would suggest that NOX4 might mediate the regulation of MKP2 by TGF-β and the over-activation of the RAS/MEK/ERK pathway in liver tumor cells would interfere with this effect. Furthermore, in HepG2 cells, as observed in fetal rat hepatocytes (Murillo et al. 2005) and hepatoma cells (as discussed in the previous section), TGF-β is up-regulating anti-apoptotic genes, such as the members of the BCL-2 family BCL-XL and MCL1 (Fig. 25). Interestingly, a significant decrease in the expression of these genes was observed after cell treatment with TGF-β and the MEK/ERK inhibitor, and NOX4 is required for this effect (Figs. 25 and 29). The consequence of all these changes in the expression pattern of the BCL-2 family is an increase in the percentage of cells presenting active BAX or BAK, a decrease in the mitochondrial transmembrane potential and an activation of caspase-3, all these events being blocked when NOX4 is targeted knock-down (Figs. 25, 28 and 29). Finally, the use of a NADPH oxidase inhibitor alone (DPI) or in combination with a permeable form of GSH (GEE) completely abrogates ROS production, the changes in BCL-2 family expression and caspase-3 activation induced by the co-treatment with TGF-β and PD98059 (Fig. 30), once more indicating the importance of ROS for TGF-β-mediated apoptosis.

Apoptosis is not only mediated by the mitochondria, it has also been shown that cell death can be promoted by other organelles such as the lysosoma and the endoplasmatic reticulum. NOX4 activity has been localized in intracellular membranes, such as endoplasmic reticulum (Chen et al. 2008), and preferentially originates hydrogen peroxide as a product (Martyn et al. 2006). Upon HepG2 cell stimulation with TGF-β and the MEK/ERK inhibitor, up-regulation of NOX4 in endoplasmic reticulum might generate ROS which would affect the correct translation/folding and/or oxidative status of different proteins, which might have consequences on transcription and translation of, at least, some apoptosis regulatory genes. Due to this intracellular localization, we wondered whether NOX4 might be affecting mechanisms of cell death promoted by other organelles, such as lysosomal-dependent events. Lysosomal permeabilization is a key feature of hepatocyte lipotoxicity inducing cell death in a BAX dependent manner (Anan et al. 2006), or in response to TNF-α (Werneburg et al. 2002). Interestingly, the co-treatment with TGF-β and the MEK/ERK inhibitor induces loss of lysosomal membrane potential (Fig. 23). We suggest that a cathepsin B-mediated mechanism might play a role in the TGF-β induced HepG2 cell death (Fig. 31) when MEK/ERK is inhibited, but this should be a NOX4-independent event, as NOX4 silencing is unable to abrogate disruption of lysosomal membrane potential (Fig. 28).

In summary, results presented here indicate that over-activation of the MAPK/ERK pathway in liver tumor cells might play a role in the initiation and/or development of HCC, through conferring resistance to the apoptosis induced by the physiological regulator TGF-β. As recently proposed, liver cancer stem cells might also exhibit relative resistance to TGF-β-induced apoptosis associated to up-regulation of the MAPK pathway (Ding et al. 2009). Here we show that inhibition of the MEK/ERK pathway might switch the liver tumor cell response to TGF-β, recovering the efficient signaling in terms of apoptosis and preventing other cell responses that would favour tumor progression. The key

molecule in this suppressor arm should be NOX4, responsible for regulating the expression of members of the BCL-2 family, which finally govern mitochondrial-dependent events (Scheme 2). The absence of standard systemic therapy for advanced cases of HCC has changed with the recent positive randomized trial testing the multikinase sorafenib, which represents a breakthrough in the management of this neoplasm (Llovet et al. 2008). It is worthy to point out that sorafenib induces tumor cell apoptosis in HCC cells, through, at least, inhibiting the RAF/MEK/ERK pathway (Liu et al. 2006). Future work will be necessary to analyze whether these new targeted drugs might be promoting the HCC response to TGF-β in terms of apoptosis favouring tumor regression.



Scheme 2. Over-activation of MEK/ERK pathway impairs TGF-β induced mitochondrial-dependent apoptosis, avoiding NOX4 up-regulation

3. Effects of TGF-β on liver cell phenotype and differentiation state.

The epithelial-mesenchymal transition (EMT), a developmental process by which epithelial cells reduce intercellular adhesion and acquire fibroblastoid properties, has been shown to be critical for the development of the invasiveness and metastatic potential of human cancers (Thiery et al. 2009). Cells undergoing EMT lose expression of E-cadherin and other components of epithelial cell junctions. Instead, they up-regulate mesenchymal markers (e.g. vimentin and N-cadherin), produce a mesenchymal cell cytoskeleton and acquire motility and invasive properties (Acloque et al. 2009). This process is initiated by the major EMT regulators such as Snail, Slug, and Twist (Peinado et al. 2004; Peinado et al. 2007). The clinical relevance of EMT has been confirmed in certain types of human cancer and the significance of individual EMT regulators in HCC has been demonstrated as EMT often precedes cell migration and invasion (Miyoshi et al. 2005; Yang et al. 2009).

TGF-β promotes EMT by a combination of Smad dependent transcriptional events and Smad-independent effects on cell-junction complexes (Padua and Massague 2009). The present study shows that TGF-β induces an EMT process in those cells that induce survival signals through EGFR in response to this cytokine: FaO rat hepatoma cells (Figs. 32-33), human fetal hepatocytes (Figs. 35), and Hep3B cells (Fig. 39). In the three models studied, TGF-β induces the expression of Snail which correlates with a decrease in E-cadherin expression, together with an increase in the expression of the mesenchymal markers Vimentin, SMA and/or N-cadherin. Moreover, cells acquire a fibroblastoid morphology and reorganize their F-actin cytoskeleton into lamellipodia, ruffles and stress fibers, events known to play an important role in migration and invasion (Friedl and Wolf 2009; Xu et al. 2009). Our group first described that fetal rat hepatocytes not only respond to TGF-β inducing apoptosis, but also undergoing EMT (Valdes et al. 2002), a fact that was later confirmed in mouse hepatocytes and AML12 cells (Kaimori et al. 2007; Dooley et al. 2008). In the present work we highlight the relevance that this process might also have during human liver development and tumorigenesis.

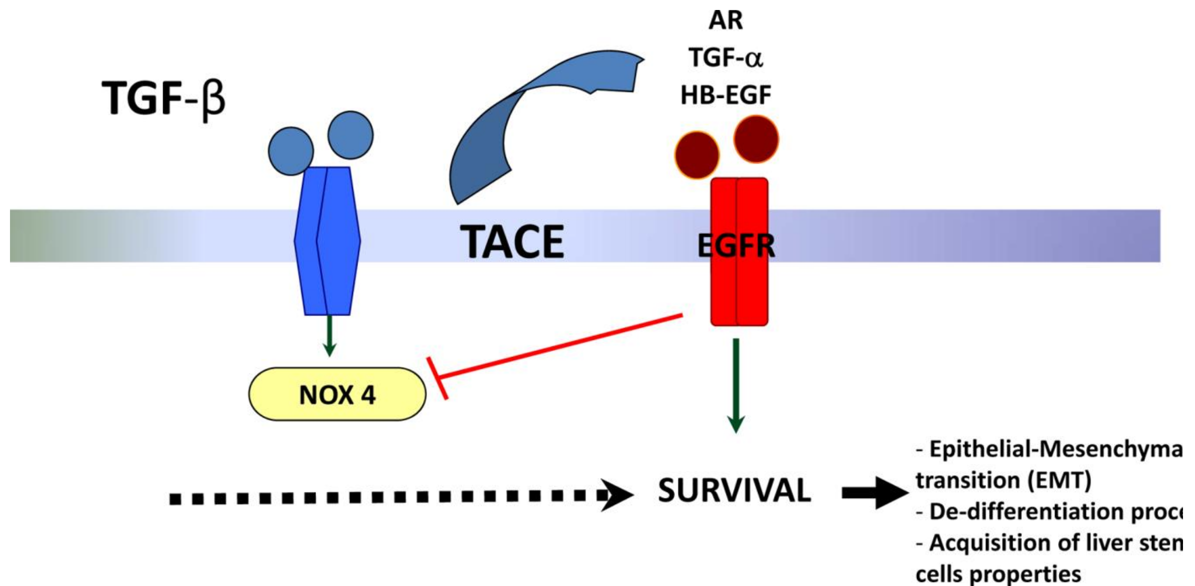
Several concerns arise when trying to study EMT in a primary hepatocyte culture, the most critical being contamination with resident fibroblasts and/or stellate cells/myofibroblasts. To ensure that the fibroblastoid population obtained after treating HFH with TGF-β was originated from hepatocytes, IHC was performed to investigate the expression of several hepatic markers (Albumin, AFP, Cx26, Hepar1, α1-antitrypsin and CK18). We observed that after 72 hours of treatment, transdifferentiated cells still express these markers (Fig. 36), even though at a lower level than untreated cells, but after longer exposures the expression of some of these markers is very low, o nearly lost (Hepar1 and CK18) (Fig. 37). Our group has previously described that TGF-β promotes a de-differentiation process in rat fetal hepatocytes (Sanchez et al. 1999; Valdes et al. 2002; del Castillo et al. 2008). In this work, we show that TGF-β induced EMT is accompanied with a de-differentiation process in the two liver tumor cell lines studied and in a non-transformed model, such as human fetal hepatocytes. This process is sustained by the loss in the expression of hepatic proteins, the diminished expression of liver enriched transcription factors that are expressed in mature hepatocytes, such as HNF4α or HNF1α, and the increase in the expression of liver transcription factors characteristic of early embryonic stages, such as HNF3β (Figs. 34, 36, 37 and 40). Moreover, it is worthy to note that in the three cell models analyzed there is an increase in the expression of CK7, Thy1, c-Kit and/or EpCAM, proteins that different groups have described as markers of hepatic stem cells (Libbrecht and Roskams 2002; Dan et al. 2006; Li et al. 2006b; Schmelzer et al. 2007; del Castillo et al. 2008; Yang et al. 2008d), indicating that TGF-β-induced EMT is coincident with an increase of stem cells markers. Our results are in agreement with recent published evidence that induction of EMT in normal or neoplastic mammary epithelial cells result in the enrichment of a population with stem-like properties (Mani et al. 2008; Morel et al. 2008). Furthermore, TGF-β also induces the self-renewal capacity of patient-derived gliomas-initiating cells and prevents cell differentiation, independently of the induction of an EMT process (Penuelas et al. 2009). Further investigation is required to prove that TGF-β-treated HFH, FaO or Hep3B cells have self-renewal properties, and the ability to seed tumors at limiting dilution, but, at least, here we show that it is possible to reverse the transdifferentiation process induced by TGF-β in human fetal hepatocytes when cells are cultured in the presence of a rich differentiation medium (Fig. 38). This finding shows great physiological and pathological relevance: on the one side, it indicates that after undergoing EMT, human cells might repopulate the liver and re-differentiate to mature hepatocytes, which might have interest in liver transplantation; on the other side, in liver tumorigenesis, the mesenchymal-epithelial transition (MET, the reversal process of

EMT) seems to be required to form metastatic nodes once the primary cells have migrated (Polyak and Weinberg 2009).

Different data in the literature support the idea that liver progenitors might show mesenchymal phenotype and suffer a mesenchymal-epithelial transition when differentiated into hepatocytes or bile duct cells. Strick-Marchand et al. (2004) isolated bipotential mouse embryonic liver cell lines from wild-type mice with two morphologies: epithelial and mixed. The mixed morphology lines contained cells that showed palmate-like morphology at low density and could be induced to differentiate into either hepatocytes, when cultured on floating aggregates, or bile duct cells, when cultured on Matrigel. Interestingly, recent results also indicate that human fetal liver multipotent progenitor cells (hFLMPC) are mesenchymal epithelial transitional cells, probably derived from the mesoendoderm (Dan et al. 2006), and show simultaneous expression of CKs and vimentin. When placed in appropriate media, these cells differentiate into hepatocytes and bile duct cells, but also into fat, bone and other cells of mesenchymal lineages. Furthermore, multipotent adult progenitor cells from bone marrow showing mesenchymal phenotype may be differentiated into functional hepatocyte-like cells (Schwartz et al. 2002). All these results together and data here presented indicate that mesenchymal-epithelial transitions might play important roles in liver differentiation from putative stem cells. Here we demonstrate that reversion of differentiated liver cells from an epithelial to a mesenchymal phenotype, such as that induced by TGF-β, might allow the enrichment in a population of cells with putative liver progenitor properties. Loss of the differentiated phenotype, concomitant with the acquisition of mesenchymal and stem cell properties, in human liver cells might indicate that the origin of cancer stem cells in HCC would occur through a de-differentiation process in addition to the mobilization of a stem cell compartment. The source could be either normal or pre-malignant hepatocytes, which could later contribute to the initiation or progression of both fibrogenesis and hepatocarcinogenesis. The origin of cancer stem cells in HCC from hepatic progenitor cells cannot be ruled out since more and more articles support this idea (Durnez et al. 2006; Lee et al. 2006a; Tang et al. 2008; Yamashita et al. 2008b). Additionally, it is worthy to point out that the phenotypic characteristics observed in the TβT-HFH (elongated phenotype, strong expression of Thy-1, Vimentin or SMA) are also highly reminiscent of myofibroblasts (Dudas et al. 2007). These cells are activated in vivo by fibrogenic cytokines, such as TGF-β, and play essential roles in liver fibrosis (Bataller and Brenner 2005). For years, it was thought that the source of myofibroblast were hepatic stellate cells (HSCs) and local mesenchymal cells recruited from the bone marrow (Kalluri and Neilson 2003), but emerging evidence suggests that the major source of fibrosis- and tumor-associated myofibroblasts in the liver might also appear through transdifferentiation from non-malignant epithelial or epithelial-derived carcinoma cells through EMT (Radisky et al. 2007; Zeisberg et al. 2007c; Dooley et al. 2008; Nitta et al. 2008). However, this is a controversial issue since a recent publication strongly disagrees with the possibility that hepatocytes can undergo an EMT process *in vitro* or *in vivo* and contribute to collagen production in liver fibrosis (Taura et al. 2009). Results here presented strongly support the idea that human hepatocytes and hepatocellular carcinoma cells may undergo EMT in response to TGF-β and could contribute to fibrosis through a transdifferentiation process.

In summary, our results support the previous postulated hypothesis that hepatocytes can also function as facultative stem cells (Michalopoulos et al. 2005) and demonstrate that TGF-β might play an essential role in the transdifferentiation process. These findings have implications for regenerative biology of the liver and open new perspectives for the ''in vitro'' isolation of putative liver stem cells to be used in basic and translational research in the liver field. Results presented here also suggest that the fibroblastic-like phenotype produced by TGF-β could undergo transdifferentiation to an epithelial phenotype, which might be of interest in new approaches for anti-fibrotic therapy. Finally, results

indicate that chronic expositions of liver tumor cells to TGF-β might modulate the cell response to this factor, switching from tumor suppression to tumor progression, through EMT processes that produce increase and cell migration and transdifferentiation to a mesenchymal, stem-cell like phenotype (Scheme 3).



Scheme 3. Cells that survive TGF-β cytotoxic effects, respond to this cytokine favouring fibrogenesis and hepatocarcinogenesis

Highlighting the importance of TGF-β in both fibrogenesis and hepatocarcinogenesis, various experimental approaches have addressed the potential of blocking TGF-β signal transduction, showing that fibrogenesis may be counteracted by the use of adenoviral overexpression of antagonistic Smad7 protein or dominant-negative or soluble TGF-β receptor (Nakao et al. 1999; Qi et al. 1999; Nakamura et al. 2000; Dooley et al. 2003). Interestingly, very recent works have indicated that inhibition of the TGF-β receptor I up-regulates E-cadherin and attenuates migration/invasion of HCC cells (Fransvea et al. 2008), inhibits neoangiogenesis (Mazzocca et al. 2009b) and targets the crosstalks between HCC and the stroma (Mazzocca et al. 2009a). Our results open new perspectives about the molecular mechanisms mediated by TGF-β that might explain the effectiveness of the inhibitors of the TGF-β pathway in impairing liver tumor fibrosis and tumor progression and invasiveness.

IX. CONCLUSIONS

First. Liver cells that are sensitive to TGF-β-induced apoptosis respond to this cytokine through inducing the expression of the NADPH oxidase NOX4, concomitant with reactive oxygen species (ROS) production. Impairment of any of these two events abrogates TGF-β induction of cell death. NOX4 expression is required for an efficient mitochondrial-dependent apoptosis.

Second. TGF-β not only induces apoptosis in human fetal hepatocytes, or liver tumor cells, but also promotes survival signals through the activation of the Epidermal Growth Factor Receptor (EGFR) pathway. TGF-β activation of EGFR counteracts its apoptotic effects at two different levels: on the one hand, EGFR activation attenuates the up-regulation of NOX4 and some pro-apoptotic members of the BCL-2 family, such as BMF, BIM or BIK; on the other hand, EGFR pathway enhances the expression of some anti-apoptotic proteins, particularly BCL-XL, MCL1 and XIAP or HIAP1, depending on the cell type.

Third. Targeting knock-down of the EGFR pathway, or inhibition of its kinase activity, potentiates TGF-β-induced apoptosis specifically in liver tumor cells that show certain sensitivity to TGF-β-induced cell death. However, some cell lines, such as HepG2 or SK-Hep1 cells, which are completely resistant to TGF-β cytotoxicity and do not up-regulate NOX4, are not sensitized with EGFR inhibitors, indicating that some survival pathways, downstream from the EGFR, might be impairing TGF-β signals.

Fourth. Overactivation of the RAS/MEK/ERK pathway impairs TGF-β-induced apoptosis in liver tumor cells. MEK inhibition sensitizes those cells to respond to TGF-β through inducing NOX4 expression and increasing ROS production.

Fifth. Targeting knock-down of NOX4 with specific siRNA has revealed that activation of this NADPH oxidase is required for the up-regulation of BMF and BIM that takes place in response to the combined treatment of TGF-β and the MEK inhibitor, as well as for impairing TGF-β-induced up-regulation of BCL-XL and MCL1.

Sixth. Both human untransformed hepatocytes, as well as liver tumor cells, are susceptible to respond to TGF-β inducing a reversible epithelial to mesenchymal transition process, which is coincident with loss of the differentiated phenotype and acquisition of stem cell markers.

CONCLUDING REMARK

Overactivation of survival signals in human liver cells, either mediated by TGF-β itself, or by molecular alterations concomitant with the tumorigenic process, switches the TGF-β role from tumor suppressor to tumor promoter, through imparing cell death and promoting epithelial-mesenchymal transitions and acquisition of a stem cell-like phenotype.

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