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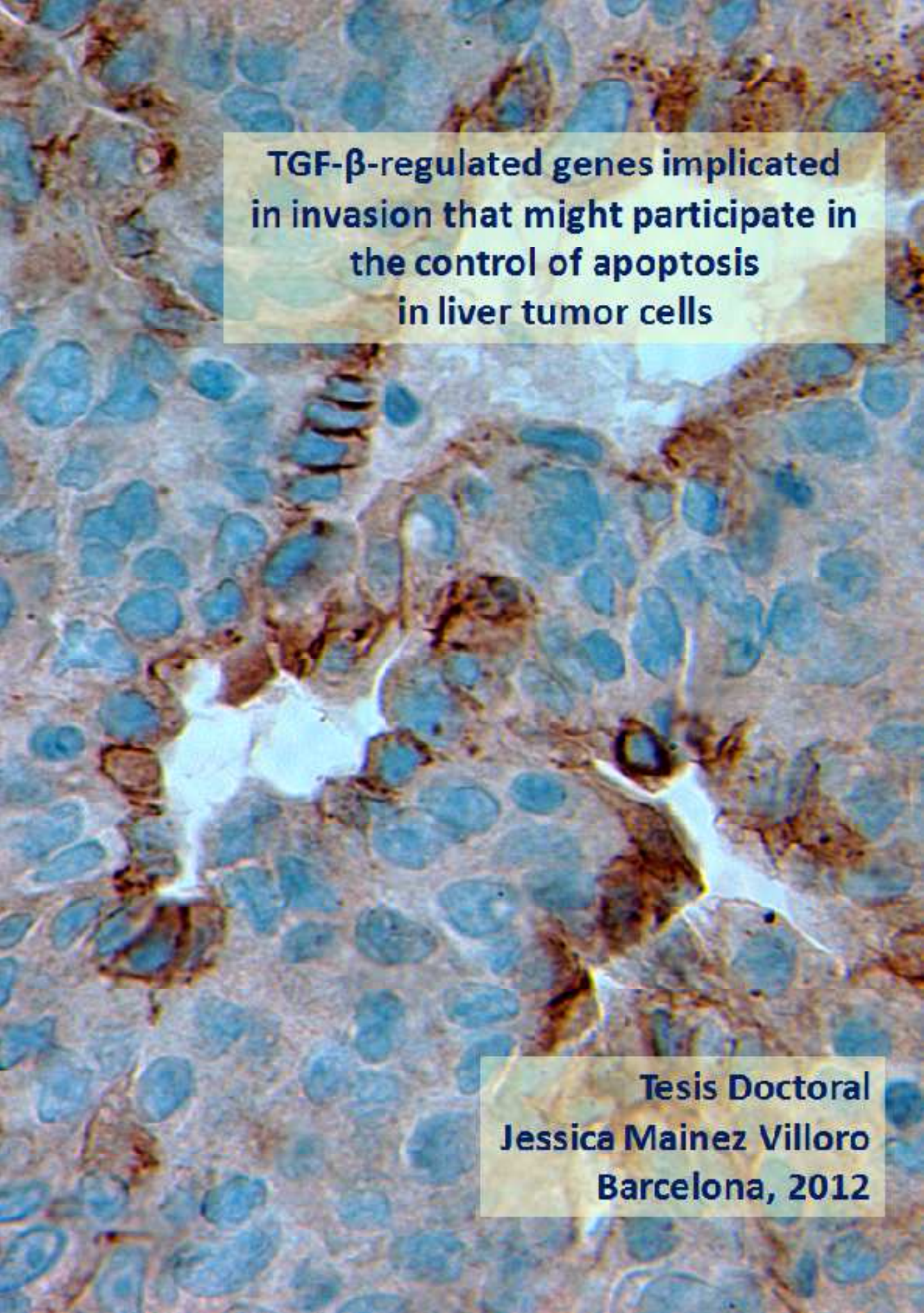

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“TGF-β-regulated genes implicated in invasion that might participate in the control of apoptosis in liver tumor cells”

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ABBREVIATIONS

AFP	Alfa-Fetoprotein
α-SMA	Alpha-Smooth Muscle Actin
AIF	Apoptosis inducing factor
ALB	Albumin
ALK	Activin Receptor-Like Kinase
Bcl2l1	Bcl-xL
BH	Bcl-2 Homology
BMP	Bone Morphogenic Protein
CARD	Caspase-recruitment domain
CC	Cholangiocarcinoma
CDH1	E-Cadherin
CHC	Combined hepatocellular-cholangiocarcinoma
CK, Krt	Citokeratin
CLC	Cholangiolocellular carcinoma
CLHCC	cholangiocarcinoma-like HCC
CSCs	Cancer stem cells
DAPK	Death associated protein kinase
DIABLO	Direct inhibitor of apoptosis protein (IAP)-binding protein with low PI
DISC	Death-Inducing Signaling Complex
DLK1	Delta-like 1 homolog
ECC	Extrahepatic cholangiocarcinoma
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal growth factor receptor
EMT	Epidermal to Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ERK	Extracellular Signal-Regulated Kinase
FaO-D	Culture derived from FaO-induced tumors
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FSP1	Fibroblast-specific protein 1
GDFs	Growth and differentiation factors
GSK3β	Glycogen synthase kinase 3 beta
HB	Hepatoblastoma
HB-EGF	Hepatin-Binding EGF
HGF	Hepatocyte Growth Factor
HNF	Hepatocyte Nuclear Factor
HPC	Hepatic progenitor cell
IAP	Inhibitor-Of-Apoptosis Protein

ICC	Intrahepatic cholangiocarcinoma
IGF2	Insulin-like growth factor 2
JNK	C-Jun N-Terminal Kinasa
Kit	c-kit receptor
MAPK	Mitogen-Activated Protein Kinase
MET	Mesenchymal–Epithelial Transition
MH	Immortalized neonatal murine hepatocytes
MH1	Mad-homology 1
MIF	Müllerian inhibitory factor
MIG6	Mitogen inducible gene 6
miRNAs	MicroRNAs
MMP	Matrix metalloproteinase
MOMP	Mitochondrial Outer-Membrane Permeabilization
MSK1	Mitogen- and stress-activated kinase 1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NES	Nuclear export signal
NF-κB	Nuclear Factor-Kappab
NLS	Nuclear localization signal
NOX	NAPDH Oxidase
OLT	Orthotopic liver transplantation
OMM	Outer mitochondrial membrane
PDGF	Platelet-Derived Growth Factor
PI3K	Phosphoinositide-3-Kinase
PTEN	Phosphatase and tensin homolog
ROS	Reactive Oxygen Species
RT	Room temperature
SARA	Smad anchor for receptor Activation
SD	Standard Deviation
SEM	Standard Error of Mean
Shc	Src homology domain 2- containing protein
siRNA	Small interfering RNA
Smac	Second mitochondria derived activator of caspase
Snai1	Snail
Snai2	Slug
SPARC	Secreted protein acidic and rich in cysteine
STAT	Signal Transducers And Activators Of Transcription
TACE/ADAM17	TNF-Alpha Converting Enzyme
TAK1	TGFβ-associated kinase 1
TGF-α	Transforming Growth Factor-Alpha

TGF-β	Transforming Growth Factor-Beta
TNF	Tumor necrosis factor
TNFRI	TNF receptor 1
TRAF6	Tumor necrosis factor receptor (TNFR)-associated factor 6
TRAIL-R1	TNF-related apoptosis-inducing ligand-receptor 1
TRAIL-R2	TNF-related apoptosis-inducing ligand-receptor 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TβRI	TGF-Beta Receptor I
TβRII	TGF-Beta Receptor II
TβT-FaO	TGF-Beta-Treated FaO rat hepatoma cells
TβT-MH	TGF-Beta-Treated immortalized murine hepatocytes
TβT-R	TβT-FaO-induced tumor with bloody appearance
TβT-R-D	Culture derived from TβT-R tumors
TβT-W	TβT-FaO-induced tumor with white appearance
TβT-W-D	Culture derived from TβT-W tumors
VEGF	vascular endothelial growth factor
Vim	Vimentin

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I. SUMMARY

In the last years our research has been focused on analyzing the signaling pathways induced by TGF-β in liver tumor cells, to understand the molecular mechanisms that confer resistance to its suppressor effects. TGF-β induces apoptosis in fetal and neonatal murine hepatocytes, as well as in liver tumor cells, and chronic exposure of these cells to TGF-β induces a process of Epithelial to Mesenchymal Transition (EMT).

In the present work we wanted to identify TGF-β-regulated genes that being involved in EMT and cell invasion could also participate in the control of growth, apoptosis and/or differentiation. Firstly we analyzed the role of genes regulated by TGF-β and implicated in EMT that might participate in apoptosis control in hepatocytes, focusing on Snail and SPARC. Inhibition of Snail, through targeting knock-down with specific siRNA, impairs TGF-β-induced EMT in murine hepatocytes and significantly enhances their apoptotic response, which indicates that Snail plays a relevant role in conferring resistance to TGF-β-induced cell death. TGF-β also induces anti-apoptotic signals, mediated by the activation of the epidermal growth factor receptor (EGFR). Snail downregulation impairs the TGF-β-induced EGFR ligands expression and inhibits the phosphorylation of Akt, Erks and c-Src family, which is coincident with activation of mitochondrial-dependent apoptotic events and an earlier Smad3 phosphorylation in TGF-β-treated cells. We also demonstrate a role for Snail in sensitizing murine hepatocytes to cell death by anoikis, which is a relevant phenomenon in metastatic processes. Snail1 downregulation in human hepatocellular carcinoma (HCC) cells, which are partially or fully resistant to TGF-β suppressor effects, restores the apoptotic response to TGF-β. TGF-β induces SPARC expression in FaO rat liver tumor cells but not in neonatal murine untransformed hepatocytes. SPARC inhibition, through targeting knock-down with specific siRNA, reveals a role for SPARC in mediating TGF-β-induced EMT in liver tumor cells. Furthermore, SPARC knock-down significantly enhances the TGF-β-induced apoptotic response. Interestingly, SPARC effects might be mediated by Snail, since SPARC silencing impairs Snai1 up-regulation by TGF-β in FaO cells.

We next wanted to study the tumorigenesis of FaO cells after *in vitro* chronic treatment with TGF-β for 4 weeks (TβT-FaO). For this, we injected these cells through both subcutaneous and intrasplenic procedures. Liver tumor formation derived from intrasplenic injection of FaO cells induced a multifocal highly proliferative hepatocarcinoma in all mice, whereas parallel inoculation of TβT-FaO cells promoted low proliferative unifocal and heterogeneous hepatic lesions which showed higher staining for phospho-Smad2. Detailed analysis of tumors revealed lesions with bile duct characteristics and lesions with a dedifferentiated (hepatoblast) phenotype. Primary culture of tumor cells from both FaO- and TβT-FaO-induced intrasplenic lesions indicated that only cells obtained from FaO-induced tumors undergo apoptosis in response to TGF-β, whereas TβT-FaO-derived tumors contain cells that are fully resistant. Analysis of the phenotype of tumors and their derived cells showed that intrasplenic injection of TβT-FaO cells may produce cholangiocarcinoma-like and hepatoblastoma-like tumors. In summary, chronic *in vitro* TGF- β treatment of FaO cells

changed their tumorigenic potential. Tumor growth was slower but cells are resistant to apoptosis. Futhermore, phenotype of lesions reflected a stem-like phenotype which provokes the appearance of less differentiated tumors (hepatoblastomas) or transdifferentiation to a different liver tumor lineage (cholangiocarcinomas).

II. RESUMEN EN ESPAÑOL

INTRODUCCIÓN

Apoptosis

La apoptosis es una forma de muerte celular programada vista en metazoos, caracterizada por la formación de burbujas en la membrana y la condensación de la cromatina entre otras características morfológicas. Es un proceso estrechamente regulado y conservado durante la evolución y es esencial para el desarrollo y el mantenimiento de la homeostasis de los tejidos (Youle and Strasser 2008, Chowdhury et al. 2006). Las proteínas principales involucradas en la apoptosis son las caspasas y la familia Bcl-2.

Las caspasas (cisteín-aspartato proteasas) son una multifuncional y altamente regulada familia de enzimas que catalizan una serie de reacciones biológicas como la apoptosis y otros eventos de remodelación celular (Crawford and Wells 2011). Basándose en su función las caspasas se clasifican en caspasas inflamatorias (caspasa-1, -4 y -5) que están implicadas en la maduración de citoquinas y en la inmunidad innata y caspasas que regulan la apoptosis. Éste grupo se divide en dos, encontrándose las caspasas iniciadoras (caspasa-2, -8, -9 y -10) y efectoras o ejecutoras (caspasa-3, -6 y -7) (Olsson and Zhivotovsky 2011). Como muchas otras proteasas, las caspasas se sintetizan como zimógenos y necesitan ser proteolizadas para activarse (Khan and James 1998). Las caspasas iniciadoras son activadas tras el reclutamiento en complejos de apoyo y luego cortan y activan las caspasas efectoras. Éstas se producen de forma constitutiva en las células como dímeros y una vez activadas actúan sobre un amplio rango de proteínas celulares que llevan a la muerte celular (Boatright and Salvesen 2003).

La familia de proteínas Bcl-2 comparten uno o varios dominios BH (homología Bcl-2) y tienen tanto una función pro- como anti-apoptótica (Danial 2007) en la vía intrínseca de apoptosis. Los miembros pro-supervivencia de esta familia incluye Bcl-2, Bcl-w, Bcl-xL, Mcl-1 y A1 (Strasser 2005). Las proteínas BH3-only son pro-apoptóticas y entre sus miembros se incluyen Bad, Bid, Bik/NBK, Bim/Bod, Bmf, Hrk/DP5, Noxa y Puma/BBC3 (Willis and Adams 2005). La apoptosis regulada por la familia Bcl-2 necesita de Bax o Bak para el completo daño mitocondrial, son sus mayores efectores. Después del inicio de la cascada apoptótica Bax y Bak oligomerizados forman un canal o un poro en la membrana externa de la mitocondria para permitir la salida de citocromo c y Smac/DIABLO (Annis et al. 2005).

Existen dos vías principales de apoptosis, la vía extrínseca mediada por receptores de muerte y la vía intrínseca o también llamada mitocondrial (Fulda and Debatin 2006).

Los receptores de muerte son miembros de la familia del factor de muerte tumoral (TNF) que incluyen más de 20 proteínas. Éstas comparten un dominio extracelular similar rico en cisteína y uno citoplasmático de unos 80 aminoácidos llamado dominio de muerte que tiene un papel crucial en la transmisión de la señal de muerte desde la superficie celular hacia rutas de señalización intracelulares. Los receptores de muerte

mejor caracterizados son CD95 (APO-1/Fas), receptor 1 de TNF (TNFRI), receptor 1 ligando inductor de TNF (TRAIL-R1) y TRAIL-R2, mientras que el papel del receptor de muerte 3 (DR3) o el de DR6 no han estado todavía bien definidos (Walczak and Krammer 2000). Los correspondientes ligandos de la superfamilia del TNF comprende ligandos de receptores de muerte como el ligando de CD95 (CD95L), TNFα, linfotoxina-α (estos últimos se unen a TNFRI), TRAIL y TWEAK, un ligando para DR3 (Walczak and Krammer 2000). Esta familia tiene un amplio rango de funciones biológicas incluyendo la regulación de la muerte celular y supervivencia, diferenciación o regulación inmune (Ashkenazi 2002, Walczak and Krammer 2000).

La vía mitocontrial se inicia por la liberación de factores apoptogénicos como citocromo c, factor inductor de apoptosis (AIF), Smac (segundo activador de caspasas derivado de mitocondrias)/DIABLO (proteína de unión directa a IAPs (proteínas inhibidoras de la apoptosis) con bajo pI) Omi/HtrA2 o la endonucleasa G desde el espacio intermembrana mitocondrial (Saelens et al. 2004, Cande et al. 2004). La liberación de citocromo c en el citosol desencadena en la activación de la Caspasa-3 a través de la formación del apoptosoma, complejo que contiene citocromo c/Apaf-1/Caspasa-9 (Bratton et al. 2001, Cain et al. 2000); mientras que Smac/DIABLO y Omi/HtrA2 promueven la activación de caspasas a través de la neutralización de los efectos inhibidores de las IAPS (Du et al. 2000, Verhagen et al. 2000). También es posible la ejecución de la muerte celular por mecanismos independientes de caspasas (Saelens et al. 2004).

La transición Epitelio-Mesénquima (EMT)

La transición Epitelio-Mesénquima (EMT) es un cambio transitorio y reversible de un fenotipo epitélico, polarizado a uno fibroblástico o de célula mesenquimática que exhibe propiedades invasivas y motiles (Grunert et al. 2003, Thiery and Sleeman 2006). Durante la EMT, los complejos de adhesiones adherentes se desensamblan y el citoesqueleto de actina se reorganiza de un alineamiento epitelial cortical asociado con uniones célula-célula hacia fibras de estrés que están ancladas a los complejos de adhesiones focales (Chung and Andrew 2008).

El fenotipo mesenquimático se caracteriza por la expresión de proteínas citoesqueléticas mesenquimáticas como la Vimentina y un aumento de la deposición de proteínas de la matriz extracelular, incluyendo colágenos y fibronectina. Estos componentes de la matriz extracelular estimulan la señalización por integrinas e inducen la formación de complejos de adhesiones focales, que facilitan la migración celular (Miettinen et al. 1994, Imamichi and Menke 2007). Estos eventos tienen como consecuencia la pérdida de la polaridad apical-basal que es crítica para el mantenimiento de la morfología y funcionalidad epiteliales (Grunert et al. 2003) y la adquisición de una polaridad frontal-posterior que les permite migrar de manera direccional (Moustakas and Heldin 2007).

La EMT puede iniciarse por diferentes moléculas señalizadoras como el factor de crecimiento epidérmico (EGF), el factor de crecimiento de hepatocitos (HFG), el factor de crecimiento transformante β (TGF-β), proteínas morfogenéticas del hueso (BMPs) Wnts y Notch (Barrallo-Gimeno and Nieto 2005). Diversas familias de factores de transcripción, incluida las familias Snail, ZEB y bHLH (hélice-bucle-hélice básica) actúan coordinados para controlar el proceso de EMT y los cambios en los patrones de expresión génica inherentes a la EMT (Peinado et al. 2007, Moreno-Bueno et al. 2008b).

Se han descrito tres tipos de EMT (Kalluri and Weinberg 2009). La EMT tipo 1 es la asociada con la implantación, formación del embrión y desarrollo de órganos y ocurre durante un proceso autónomo que tiene implícita la necesidad de generar diferentes tipos de células que comparten fenotipo mesenquimático y biomarcadores (Thiery 2002, Thiery and Sleeman 2006). La EMT tipo 2 es la asociada con la cicatrización de heridas, la reparación de tejidos y la fibrosis de órganos (Kalluri and Weinberg 2009, Zeisberg and Neilson 2009). El mecanismo de EMT incrementa el fenotipo migratorio y genera fibroblastos para reconstruir y reparar el tejido tras un trauma y/o una lesión inflamatoria (Bissell and Radisky 2001) aunque puede continuar la inflamación y llevar a la fibrosis y la destrucción del órgano (Kalluri 2009). La EMT tipo 3 ocurre en células cancerígenas epiteliales que difieren genética o epigenéticamente de las células epiteliales no transformadas. Las células generadas por esta EMT pueden invadir y metastatizar a través del sistema circulatorio y generar manifestaciones sistémicas de una progresión cancerosa maligna (Kalluri 2009).

El proceso de EMT se ha reconocido como un paso crucial en la cascada metastásica (Massague 2008, Thiery et al. 2009, Polyak and Weinberg 2009), correlacionando con invasividad, mal pronóstico y cáncer más agresivo (Massague 2008, Sarrió et al. 2008). Las células de carcinoma que adquieren un fenotipo mesenquimático se muestran principalmente en el frente de los tumores primarios y son consideradas las células que entrarían en los subsiguientes pasos de la cascada de invasión metastásica: intravasación, transporte a través de la circulación, extravasación, formación de micrometástasis y colonización (Brabletz et al. 2001, Thiery 2002, Fidler and Poste 2008). Para el establecimiento de colonias secundarias en lugares distantes del tumor principal, las células cancerígenas deben perder su fenotipo mesenquimático y realizar el proceso inverso (MET, Transición Mesénquima Epitelio) hacia un fenotipo epitelial (Zeisberg et al. 2005). La tendencia de las células cancerígenas diseminadas de sufrir este fenómeno refleja la influencia del nuevo microentorno y la ausencia de las señales que experimentaron en el tumor principal que les hicieron sufrir EMT en primer lugar (Thiery 2002, Jechlinger et al. 2002, Bissell et al. 2002).

Subrayando la importancia de la EMT en la resistencia terapéutica, se han encontrado células cancerígenas con características de célula madre en los tumores residuales después de un tratamiento quimioterapéutico estándar (Yu et al. 2007, Li et al. 2008). El proceso de EMT y la adquisición de propiedades de célula madre se han asociado a un incremento de la resistencia a apoptosis en cáncer de pecho y células de mama

(Robson et al. 2006, Yu et al. 2007) así como nosotros y otros grupos han publicado en células hepáticas a través de la acción del TGF-β (Spagnoli et al. 2000, Valdes et al. 2002, del Castillo et al. 2006, Caja et al. 2009).

Snail

Snail forma parte de una superfamilia de factores de transcripción compuesta por las familias Snail y Scratch (Nieto 2002, Barrallo-Gimeno and Nieto 2009). La familia Snail contiene Snai1, Slug (Snai2 (Cohen et al. 1998)) y Smuc (Snai3 (Katoh 2003)) y muestran un alto grado de conservación en cuanto a secuencia codificante y predicción proteica, apuntando a un papel conservado en la morfogénesis temprana (Manzanares et al. 2001, Knight and Shimeld 2001).

Para ejercer su función como represor, la importación nuclear de Snail es mediada por importinas y así forma complejos ternarios con co-represores a través del dominio Snag (Mingot et al. 2009). La unión al DNA ocurre a través de las cajas E (5'-CACCTG-3') que se encuentran en la región promotora de diferentes genes como la Cadherina E (Batlle et al. 2000, Cano et al. 2000). La regulación de la actividad de Snail es a través de modificaciones post-transcripcionales en la fosforilación de diversas serinas, oxidación de lisinas y su exportación nuclear, ubiquitinización y degradación en el proteasoma (Domínguez et al. 2003, Zhou et al. 2004a, Vinas-Castells et al. 2010).

Los genes Snail son fundamentales para el desarrollo embrionario en metazoos en procesos que implican movimientos celulares a gran escala, como la gastrulación o la formación de la cresta neural en los vertebrados (Barrallo-Gimeno and Nieto 2005, Peinado et al. 2007). La activación aberrante de Snail en adultos lleva a progresión tumoral y recurrencia de los mismos (Moody et al. 2005a, Peinado et al. 2007), a fibrosis (Boutet et al. 2006) aunque también se ha detectado expresión en condiciones benignas como el tejido normal adyacente al tumor (Pena et al. 2009) o en el desarrollo de los huesos adultos (de Frutos et al. 2007, de Frutos et al. 2009).

La expresión de Snail se ha asociado con hipoxia en cáncer ovárico a nivel de transcrito (Imai et al. 2003), así como con metástasis de los nódulos linfáticos (Cheng et al. 2001) y derrames malignos en cáncer de pecho (Elloul et al. 2005), invasividad (Sugimachi et al. 2003) y mal pronóstico en carcinoma hepatocelular (Miyoshi et al. 2005). Sin embargo, se ha señalado que los niveles de transcrito pueden no correlacionar bien con los proteicos debido a que la proteína Snail está altamente regulada y tiene una vida media de tan solo 25 minutos (Zhou et al. 2004a). A nivel de inmunohistoquímica se ha detectado en diferentes tipos de cáncer incluidos el tracto gastrointestinal superior (Usami et al. 2008, Kim et al. 2009), cabeza y cuello (Peinado et al. 2008, Yang et al. 2008b, Schwock et al. 2010) entre otros.

SPARC (proteína ácida secretada rica en cisteína)

SPARC es una proteína que forma parte de una gran familia de proteínas asociadas a la matriz extracelular secretadas. SPARC está espacialmente y temporalmente regulada

durante el desarrollo y se expresa en altas concentraciones durante la remodelación de tejidos (Bradshaw and Sage 2001b, Brekken and Sage 2001, Tartare-Deckert et al. 2001).

Es conocida su interacción con diversos componentes de la matriz extracelular como los colágenos y actúa como una molécula des-adhesiva (Murphy-Ullrich 2001), como inhibidora de la extensión celular (Delostrinos et al. 2006), como inhibidora del ciclo celular (Bradshaw and Sage 2001a, Fenouille et al. 2010) y como moduladora de la actividad de citoquinas y factores de crecimiento. También se ha encontrado SPARC en el núcleo en células embrionarias de pollo en cultivo y en células endoteliales aorticas (Gooden et al. 1999). Esta localización nuclear no es constitutiva sino que es dependiente del ciclo celular o de su concentración (Gooden et al. 1999, Yan et al. 2005).

SPARC es un importante regulador del cambio de Cadherina E a Cadherina N, incrementa la expresión de Vimentina e induce la secreción de TGF-β (Bassuk et al. 2000, Robert et al. 2006, Girotti et al. 2011). SPARC induce la expresión de Snail durante el desarrollo del melanoma y su supresión disminuye la expresión de Snail (Robert et al. 2006). Por otra parte la expresión de SPARC está regulada por Snail en células de carcinoma pulmonar (Olmeda et al. 2007) y por Snail Slug y E47 en células MDCK (Moreno-Bueno et al. 2006).

El papel de SPARC en cáncer es complejo y dependiente del origen del tumor y del entorno en el que se desarrolla. SPARC ejerce funciones supresoras en neuroblastomas, cáncer pancreático, colorectal y ovarico y en leucemia mieloide crónica entre otros (Clark and Sage 2008, Podhajcer et al. 2008). En tumores asociados con transformación mesenquimática la expresión de los niveles de SPARC es síntoma de mal pronóstico (Lien et al. 2007). Así, SPARC promueve agresividad tumoral y metástasis en cáncer de próstata (Clark and Sage 2008, Podhajcer et al. 2008) y es un importante marcador para la agresividad, metástasis y mal pronóstico en melanoma (Massi et al. 1999, Sturm et al. 2002, Alonso et al. 2007). Su papel en el carcinoma hepatocelular es ambiguo ya que ejerce como promotor tumoral (Atorrasagasti et al. 2010) o como supresor tumoral en pacientes en estado avanzado de la enfermedad (Keating and Santoro 2009).

TGF-β (Factor de crecimiento transformante β)

El primer miembro de esta superfamilia se descubrió en 1978 (de Larco and Todaro 1978) y desde entonces se han identificado más de 40 miembros, que tienen en común su estructura dimérica y la presencia en un motivo estructural de un grupo de cisteínas (Galat 2011). Entre los TGF-βs existen seis isoformas distintas con diferentes grados de homología (Santibañez et al. 2011), aunque sólo las isoformas TGF-β1, TGF-β2 yTGF-β3 se expresan en mamíferos (Lyons and Moses 1990). Todos los miembros de la familia TGF-β se unen a receptores de superficie celular serina/treonina quinasas tipos I y II (TβRI y TβRII respectivamente), los cuales forman complejos heteroméricos en la presencia del ligando. Se han descrito siete TβRIs, también llamados ALKs (quinasas

similares a receptor de activina) así como cinco diferentes TβRIIs (Santibañez et al. 2011). Además, los ligandos TGF-β pueden interaccionar con los co-receptores endoglina y betaglicano, conocidos como TβRIIIs (receptores de TGF-β tipo III) (Gatza et al. 2010, Bernabeu et al. 2009, Shi and Massague 2003).

La señalización del TGF-β por su vía canónica o Smad-dependiente, empieza con la unión del ligando y la formación de un complejo heterotetramérico formado por dos receptores tipo I y dos receptores tipo II, donde el receptor TβRII constitutivamente activo fosforila y activa a el receptor TβRI (Heldin et al. 2009, Kang et al. 2009, Massague 2008). El activado receptor serina/treonina quinasa TβRI fosforila Smad2 y Smad3, los cuales forman un hetero-oligómero con Smad4. Se translocan del citoplasma al núcleo dónde regulan la transcripción de genes diana (Schmierer and Hill 2007, Bierie and Moses 2006, Zavadil and Bottinger 2005). Existen diferentes proteínas asociadas a receptores que facilitan la interacción de las Smads con el complejo de receptores y consecuentemente su activación. Ejemplos de estos adaptadores son SARA (adaptador de Smads para la activación del receptor), una proteína que en mamíferos estabiliza la unión de las Smads2 y 3 al TβRI, la Endofina, estructuralmente homóloga a SARA para la señalización por BMPs (Shi et al. 2007b) y por TGF-β (Chen et al. 2007) y otros adaptadores de la interacción de las Smads con receptores tipo I como la Axina, Dab2 y Dok-1 (Kang et al. 2009). Las Smads pueden ser reguladas por diferentes mecanismos como la fosforilación o la degradación mediada por ubiquitinas en proteasomas y lisosomas; además de control negativo realizado por Smad7 (Heldin and Moustakas 2011).

La señalización del TGF-β por su vía no canónica o Smad-independiente, comprende las siguientes rutas de señalización como TAK1 (quinasa 1 asociada a TGF-β), Erk (quinasa regulada por señales extracelulares), p38, MAPK (quinasa activada por mitógeno) y Akt (Sorrentino et al. 2008, Yamashita et al. 2008a). También se ha demostrado que el rápido y potente efecto que tiene TGF-β en el citoesqueleto celular depende de la GTPasa Cdc42 (Moustakas and Heldin 2005, Edlund et al. 2002). Anteriormente nuestro grupo ha publicado que el TGF-β induce señales antiapoptóticas mediante la estimulación de los ligandos del receptor del EGF (EGFR) que estimula un bucle autocrino de activación del EGFR (Caja et al. 2007) y de sus efectores pro-supervivencia como son c-Src, PI3K y Akt (Valdes et al. 2004, Murillo et al. 2005).

Las señales inducidas por TGF-β están estrechamente reguladas a través de modificaciones post-transcripcionales de los componentes señalizadores, ya que esto indica su localización subcelular, actividad y duración de la señal.

El TGF-β induce señales inhibidoras de ciclo celular como los genes p15, p21 y p57 (Heldin et al. 2009, Massague 2004) y produce parada en la fase G1 temprana con lo que inhibe el crecimiento en varios tipos celulares (Massague 2004), incluidos los hepatocitos (Sanchez et al. 1995). Por otra parte, Smad2 es un regulador negativo del crecimiento de hepatocitos *in vitro* e *in vivo* (Ju et al. 2006) y Smad3 tiene un papel

como mediador de la inhibición del crecimiento del epitelio (Ju et al. 2006) aunque esto no es universalmente aplicable y su papel principal es como inductor de la EMT.

Además de parada de ciclo celular, el TGF-β induce apoptosis y sus dianas en la cascada de señalización posterior son la quinasa asociada a muerte DAPK, GADD45β (gen de parada del crecimiento e inducible por daño al ADN 45) (Jang et al. 2002, Takekawa et al. 2002, Ohgushi et al. 2005), inhibe la expresión de survivina (Wang et al. 2008) e induce la expresión de Bim y Bmf entre otros (Ramjaun et al. 2007, Caja et al. 2009). Por otra parte, la inducción de Bcl-xL junto a la represión de Bid (Ruan et al. 2010) o la rápida estimulación de Mcl-1 (Gingery et al. 2008) promueve supervivencia en osteoclastos. Del mismo modo, la inducción de la vía PI3K/Akt por TGF-β protege a las células de sus efectos pro-apoptóticos y de inhibición de ciclo celular (Shin et al. 2001, Chen et al. 1998, Valdes et al. 2004, Song et al. 2006), por la interacción de Akt con Smad3 (Shin et al. 2001, Chen et al. 1998, Song et al. 2006, Valdes et al. 2004). Por otra parte la activación de p38 MAPK tiene efectos pro-apoptóticos en células epiteliales mamarias (Yu et al. 2002) y en células B (Schrantz et al. 2001), pero no en hepatocitos (Herrera et al. 2001c). La producción de especies reactivas de oxigeno (ROS) es una de las vías de señalización utilizadas por TGF-β para inducir apoptosis (Sanchez et al. 1996), así son necesarios para la ejecución de la vía mitocondrial a través de la modulación de diferentes miembros de la familia Bcl-2 (Herrera et al. 2001b, Ramjaun et al. 2007, Kang et al. 2007). Por otra parte, la producción de ROS se bloquea por el EGF, que rescata a los hepatocitos de la apoptosis inducida por TGF-β (Carmona-Cuenca et al. 2006, Fabregat et al. 2000).

TGF-β1, TGF-β2 y TGF-β3 comparten la capacidad de inducir EMT en células epiteliales (Miettinen et al. 1994, Piek et al. 1999, Valcourt et al. 2005) en una variedad de sistemas biológicos y condiciones patológicas (Zavadil and Bottinger 2005, Bierie and Moses 2006). Así TGF-β1 y TGF-β2 tienen un papel muy importante en la cardiogénesis (Nakajima et al. 2000, Mercado-Pimentel and Runyan 2007, Azhar et al. 2003) y TGF-β3 en la formación del palatal (Nawshad et al. 2004). El papel del TGF-β en el proceso de EMT que ocurre tras el daño tisular y que contribuye a la fibrosis ha sido demostrado en fibrosis renal (Schnaper et al. 2003), pulmonar y hepática (Willis and Borok 2007, Gressner et al. 2002). El TGF-β también tiene un papel muy importante como efector clave en la EMT en progresión del cáncer y metástasis, así su expresión induce agresividad, angiogénesis y mayor migración mesenquimática en células cancerosas (Derynck et al. 2001), incluidas células cancerígenas hepáticas (Sanchez et al. 1999, Rossmanith and Schulte-Hermann 2001, Valdes et al. 2002, del Castillo et al. 2006, Caja et al. 2007, Bertran et al. 2009, Franco et al. 2010).

El papel del TGF-β en hepatocarcinogénesis es dual, ya que promueve parada de ciclo celular y apoptosis en el hígado sano y durante la iniciación del tumor y también es capaz de controlar la dediferenciación y la extensión de los hepatocitos neoplásicos a través de la inducción de EMT (Fischer et al. 2005). Las células que sobreviven a los efectos apoptóticos del TGF-β (Murillo et al. 2005, Caja et al. 2007) inducen EMT, un

proceso que media migración celular y supervivencia (Heldin et al. 2009, Valdes et al. 2002). De manera interesante, los tumores hepáticos que expresan genes relativos a una respuesta tardía al TGF-β (late TGF-β signature) muestran un fenotipo altamente invasivo y mayor recurrencia tumoral cuando se comparan con aquellos que muestran una expresión relativa al TGF-β temprana (early TGF-β signature) (Coulouarn et al. 2008). Muchos estudios han correlacionado la sobreexpresión de TGF-β1 en cáncer humano con progresión tumoral, metástasis, angiogénesis y mal pronóstico (Bierie and Moses 2006, Levy and Hill 2006) y la inhibición de la vía del TGF-β ha sido objeto de estudio como molécula diana en terapia contra el cáncer (Muraoka et al. 2002).

Mecanismos moleculares del cáncer hepático

El carcinoma hepatocelular humano (HCC) es la quinta causa de muerte por cáncer a nivel mundial (El-Serag and Rudolph 2007, Parkin et al. 2005, Sherman 2005, Kensler et al. 2003). Entre su etiología se incluyen la infección por hepatitis B o C, la intoxicación alcohólica (Farazi and DePinho 2006), la hepatitis crónica y la cirrosis resultante de la inflamación o la fibrosis entre otros factores (Kensler et al. 2003, Friedman 2008). Se han descrito múltiples causas de la proliferación aberrante y dediferenciación de los hepatocitos que llevan al HCC, como la inactivación de p53 o el aumento de la señalización de TGF-β o Wnt/β-catenina entre otros (van Zijl et al. 2009). Cerca de un 80% de los pacientes son diagnosticados en avanzados estados por falta de síntomas y tan sólo un 30-40% de pacientes son aptos para una terapia curativa, en general se considera curativa la resección del hígado y el trasplante ortotópico ya que pese a las nuevas opciones terapéuticas la no posibilidad de extirpación del tumor es de muy mal pronóstico (Llovet and Bruix 2008).

El colangiocarcinoma (CC) es un tumor maligno originado en el epitelio de los conductos biliares intra (ICC) o extrahepáticos (ECC) y su incidencia y mortalidad están incrementando a nivel mundial (Khan et al. 2003). Entre su etiología se incluyen la inflamación crónica o la estasis biliar, seguido de múltiples procesos iniciados por la liberación de factores como el TGF-β, fosforilación del EGFR o mutaciones de K-ras entre otros factores (Sempoux et al. 2011b). Es un tumor difícil de diagnosticar en estadios tempranos e incluso en casos resectables el pronóstico es muy malo (Khan et al. 2003, Sirica 2005). Su respuesta quimioterapéutica es ineficiente y la completa resección es la única terapia actual (Shimoda and Kubota 2007), pese a los avances realizados con el inhibidor multiquinasas Sorafenib (Wilhelm et al. 2008), que también se está mostrando eficaz en el tratamiento del HCC.

Existen tumores hepáticos con características mixtas entre las formas puras del carcinoma hepatocelular humano y el colangiocarcinoma. Así se han descrito el CHC (hepato-colangiocarcinoma combinado), el CLC (carcinoma colangiolocelular) y el CLHCC (hepatocarcinoma parecido a colangiocarcinoma).

El hepatoblastoma (HB) es el tumor primario hepático más común en niños, llegando hasta el 1% de todos los tumores malignos infantiles (Mann et al. 1990). Su origen es

todavía incierto aunque se sugiere una derivación del linaje hepatocelular (Calvert et al. 1995, Diwan et al. 1995) y que sus células tienen características de células bipotentes o de linaje biliar, un tipo de células hepáticas no diferenciadas (Sakairi et al. 2007). En roedores los HB se han descrito también en adultos (Frith et al. 1994) y son inducibles por productos químicos (Diwan et al. 1995). Cuando los HB son puramente fetales son resectables y curables sin quimioterapia (Weinberg and Finegold 1983), todos los otros tipos son más agresivos, necesitan resección, quimioterapia e incluso transplante (Haas et al. 2001).

ANTECEDENTES DEL GRUPO RELATIVOS AL TRABAJO DE DOCTORADO ACTUAL

El papel del TGF-β en tumorogénesis ha sido un tópico de interés en nuestro grupo. Durante los pasados años hemos explorado las diferentes vías de señalización inducidas por TGF-β en hepatocitos y en células tumorales hepáticas.

Así, hemos demostrado que el TGF-β a bajas dosis inhibe el crecimiento hepático parando las células en la fase G1 del ciclo celular (Sanchez et al. 1995) y usándose concentraciones más altas el TGF-β induce muerte celular a través de la producción de ROS (Sanchez et al. 1996) que permiten la ejecución de la vía mitocondrial de la apoptosis (Herrera et al. 2001a), a través de la modulación de diferentes miembros de la familia Bcl-2 (Herrera et al. 2001b).

Además de su capacidad de inducir apoptosis, el TGF-β induce señales anti-apoptóticas en hepatocitos fetales y en células tumorales hepáticas (Valdes et al. 2004) a través de la expresión de los ligandos del EGFR (TGF-α y HB-EGF) o la expresión de la metaloproteasa TACE, enzima responsable de su liberación (Murillo et al. 2005, Caja et al. 2007). Asimismo, la producción de ROS es bloqueada por la vía del EGF que rescata a los hepatocitos de la apoptosis inducida por TGF-β (Carmona-Cuenca et al. 2006, Fabregat et al. 2000).

Las células que sobreviven a los efectos pro-apoptóticos del TGF-β sufren EMT (Sanchez et al. 1999, Valdes et al. 2002, Caja et al. 2007). Este fenómeno induce desdiferenciación celular en hepatocitos fetales de rata (Sanchez et al. 1999, Valdes et al. 2002) e induce la expresión de marcadores de célula progenitora (Fabregat et al. 1996, Sanchez et al. 1999, Valdes et al. 2002, Caja et al. 2011a). Los hepatocitos fetales son entonces capaces de diferenciarse tanto a hepatocitos maduros como a colangiocitos (del Castillo et al. 2008, Caja et al. 2011a). El proceso de EMT confiere a las células resistencia a la apoptosis, coincidente con una mayor expresión de Bcl-xL y activación basal de Akt (Valdes et al. 2002). Esta activación temprana de Akt se puede correlacionar con la capacidad del TGF-β de transactivar las vías de c-Src y EGF en hepatocitos (Murillo et al. 2005) y mediar carcinogénesis mediante la transactivación de la vía del EGFR en células FaO (Caja et al. 2007).

Datos preliminares realizados en células de melanoma y MDCKs indican que moléculas implicadas en EMT, como Snail y SPARC, pueden regular proliferación y muerte celular (Vega et al. 2004) y la expresión de SPARC está regulada por Snail en células MDCKs (Moreno-Bueno et al. 2006, Olmeda et al. 2007).

OBJETIVOS

Objetivo general

IDENTIFICAR LOS GENES REGULADOS POR TGF-β EN CÉLULAS HEPÁTICAS QUE, ESTANDO INVOLUCRADOS EN INVASIÓN CELULAR, PUEDAN TAMBIÉN PARTICIPAR EN EL CONTROL DE CRECIMIENTO, APOPTOSIS Y/O DIFERENCIACIÓN.

Objetivos específicos

1. Análisis de genes regulados por TGF-β e implicados en EMT que puedan participar en el control de apoptosis en hepatocitos: papel de Snail y SPARC.
2. Estudio de la tumorogénesis in vivo de células tumorales hepáticas tratadas crónicamente con TGF-β in vitro: análisis morfológico y fenotípico y de la respuesta apoptótica.

RESULTADOS

Teniendo en consideración el trabajo previo de nuestro grupo decidimos explorar los efectos del TGF-β en hepatocitos neonatales murinos inmortalizados (MH) (Gonzalez-Rodriguez et al. 2008).

El tratamiento crónico de los MH con TGF-β induce un proceso de apoptosis, pero los hepatocitos que sobreviven muestran una apariencia fibloblastoide. Estas células sufren un proceso de EMT completo tal y como observamos por la reorganización del citoesqueleto de actina y aparición de fibras de estrés, la disminución de los niveles de Cadherina E y aumento de la expresión de Snail, el reemplazo de la Citoqueratina 18 por Vimentina en los filamentos intermedios, así como por la inducción del receptor de célula madre c-kit (Figuras 1 y 2).

Decidimos utilizar este modelo para estudiar los mecanismos moleculares involucrados en la EMT inducida por TGF-β que pueden conferir resistencia a sus efectos pro-apoptóticos, focalizando nuestra atención tanto en Snail como en SPARC.

Mediante el uso de siRNA, disminuimos los niveles de expresión de Snail, viendo que este hecho se traduce en una reducción de la EMT inducida por TGF-β en estas células MH (Figura 4).

La cancelación de Snail aumenta la respuesta apoptótica del TGF-β en células MH, mostrando una mayor activación de caspasa-3 y núcleos apoptóticos que lleva a la

muerte masiva a tiempos largos (Figura 5). Este hecho se ratifica en las líneas celulares humanas de hepatocarcinoma Hep3B y de adenocarcinoma hepático SK-Hep1 (Figuras 10 y 11). En la primera, la cancelación de Snail aumenta la respuesta apoptótica al TGF-β y en la segunda, la cancelación de Snail restablece la respuesta apoptótica al TGF-β que no tenía antes de la cancelación de Snail. El análisis de las señales intracelulares que median la supervivencia del TGF-β revela que tanto la inducción de los ligandos del EGFR como otras señales de supervivencia inducidas por TGF-β, como son la fosforilación de Akt, Erks y familia c-Src, se impiden al cancelar Snail1 (Figura 6). Respecto a la fosforilación de las Smads, el silenciamiento de Snail no provoca cambios en la fosforilación de Smad2 pero adelanta la de Smad3 (Figura 7), al mismo tiempo que incrementa los niveles de proteínas anti-apoptóticas de la familia Bcl-2 como Bcl-xL y Mcl-1 tanto a nivel transcripcional como post-transcripcional (Figura 8). Además de un incremento de la muerte celular, en presencia del siRNA para Snail1, se produce un adelantado y potente incremento de los niveles de los genes Bim y Bmf, previamente relacionados con la apoptosis inducida por TGF-β (Figura 8). En acuerdo con estos cambios en la expresión génica, las células expuestas al TGF-β con Bax o Bak activado aumentan en la presencia del siRNA para Snail1 (Figura 8), indicando que éste puede contrarrestar la apoptosis mitocondrial inducida por TGF-β en hepatocitos, alterando el balance de miembros pro-apoptóticos versus anti-apoptóticos de la familia Bcl-2.

También comprobamos la importancia de Snail1 en otros procesos de muerte importantes para los procesos metastásicos que se producen en la carcinogénesis como la anoikis (muerte por falta de anclaje a un sustrato). La cancelación de Snail reduce la capacidad del TGF-β de anclaje al plástico no tratado y produce menos supervivencia a la anoikis en presencia o no de TGF-β (Figura 9).

Para estudiar el papel de SPARC en la EMT y la apoptosis inducida por TGF-β cambiamos de modelo celular a la línea FaO de hepatoma de rata, ya que comparando las células MH con las células FaO, sólo la línea tumoral induce SPARC (Figura 12), asociándolo con la tumorogénesis hepática. Mediante el uso de siRNA, disminuimos los niveles de expresión de SPARC, viendo que este hecho se traduce en en una reducción de la EMT (Figura 14), la inducción de la parada de ciclo celular en G_0/G_1 y el aumento en la respuesta apoptótica inducida por TGF-β en las células FaO (Figura 15). Un hecho importante es la relación que encontramos entre Snai1-TGF-β-SPARC, señalado por la imposibilidad del TGF-β de incrementar la expresión de Snail1 cuando se cancela SPARC en células FaO (Figura 16).

Decidimos estudiar la tumorogénesis *in vivo* de las células FaO tratadas de manera crónica con TGF-β *in vitro*. Una vez aislada la línea resultante, TβT-FaO, (Bertran et al. 2009) y Figura 17), ambas se inocularon en el flanco de ratones Balb/c immunodeprimidos de manera subcutánea e intrasplénica. La primera aproximación nos permite observar que a pesar de las diferencias histológicas entre los tumores obtenidos, tienen similar tiempo de duplicación tumoral y latencia (Figura 18). La segunda aproximación nos muestra lesiones similares resultantes de la inoculación de

células FaO, un hepatoma multifocal, mientras que aparecen dos tipos de lesiones tras la inoculación de células TβT-FaO, una más pálida, que recuerda a un hepatocarcinoma o colangiocarcinoma (llamada TβT-W) y otra más irrigada que recordaba un hepatoblastoma (llamada TβT-R). La histología hecha con Hematoxilina-Eosina muestra múltiples agrupaciones de células rodeadas por tejido conectivo en las lesiones FaO mientras que se observan estructuras ductales en la lesión TβT-W y la lesión TβT-R se presenta muy desorganizada y con presencia de sinusoides (Figura 19). Las lesiones FaO son más proliferativas (por marcaje de KI67) que las lesiones TβT-FaO, correlacionando con el marcaje por TUNEL, que nos indica una compensación mediante la proliferación de hepatocitos en esta lesión. También tienen menores niveles de fosfo-Smad2 nuclear comparado con las lesiones TβT-FaO (Figura 20).

El estudio de marcadores de EMT revela que la lesión FaO muestra mayor marcaje por Cadherina E que las lesiones TβT-FaO, como las células parentales, pero también n marcaje por Vimentina que puede reflejar un proceso de EMT *in vivo* (Figura 21). El patrón de expresión de citoqueratinas (CKs) y SPARC (Figura 22), nos muestra que la inyección de las células TβT-FaO produce células con características de ducto biliar (TβT-W, positiva para CKs 18, 19 y SPARC en determinadas zonas) y con un fenotipo desdiferenciado (TβT-R, positiva para CKs 18,19 y SPARC). Las lesiones FaO sólo son positivas para CK18, como corresponde a un hepatocito maduro.

Un análisis en profundidad requiere el cultivo celular que nos permite un análisis más preciso de las lesiones. Así obtenemos los cultivos FaO-D, TβT-W-D y TβT-R-D. El cultivo FaO-D es parecido al de las células parentales FaO, así como el cultivo TβT-R-D es parecido al parental TβT-FaO. En cambio, el cultivo TβT-W-D presenta células parecidas a las parentales TβT-FaO (Figura 23) pero también es el único cultivo capaz de formar estructuras ductales cuando se siembra sobre matrigel (Figura 28). Se confirma que la velocidad de crecimiento del cultivo FaO-D es mayor que los cultivos TβT-W-D y TβT-R-D (Figura 23) y también comprobamos que es el único que induce actividad Caspasa-3 en respuesta al TGF-β (Figura 24). Los marcajes hechos por immunohistoquímica respecto a marcadores de EMT y CKs se ratifican en immunofluorescencia (Figura 25), encontrándose además que el único cultivo positivo para c-kit (marcador de célula madre pero altamente expresado en colangiocitos) es el cultivo TβT-W-D (Figura 28). El análisis de los genes relativos a EMT y diferenciación hepática de los diferentes cultivos respecto a las células parentales (Figura 27) indica que los cultivos TβT-W-D y TβT-R-D como sus células parentales FaO-TβT presentan un estado de desdiferenciación (por pérdida de Hnf4α) y aumento de marcadores de célula madre hepática como EpCam. Los marcajes de Vimentina, SPARC, c-kit y CKs 7 y 19 se ratifican con lo observado previamente, así como se analizan los niveles de Snail y Slug, encontrándose el último en todos los cultivos derivados de los tumores y pudiendo explicar la presencia de Vimentina en la lesión y el cultivo FaO-D. Es interesante observar que la re-inducción de E-Cadherina en TβT-W-D correlaciona con la baja expresión de Slug en este cultivo.

DISCUSIÓN

El TGF-β induce señales complejas y a veces opuestas en células epiteliales (Massagué, 2008; Zavadil and Bottinger, 2005; Heldin et al., 2009) haciendo difícil entender su papel específico en la progresión tumoral. En el caso del hígado, publicaciones recientes ofrecen nuevas visiones del papel del TGF-β en la hepatocarcinogénesis humana, mostrando claramente su papel supresor en estadios iniciales y su papel como promotor tumoral en estadios avanzados (Massagué, 2008). Las células cancerosas hepáticas se vuelven resistentes a la muerte inducida por TGF-β y son capaces de sufrir EMT y adquirir propiedades invasivas (Sanchez et al. 1999, Valdes et al. 2002, Valdes et al. 2004, del Castillo et al. 2006, Caja et al. 2007). De acuerdo con esto, los tumores hepáticos que expresan una firma temprana del TGF-β (genes supresores) tienen un fenotipo menos invasivo y menor recurrencia tumoral en comparación a los que expresan genes asociados a una firma tardía del TGF-β (anti-apoptóticos y metastáticos) (Coulouarn et al., 2008). De este modo es crucial entender los mecanismos que permiten a las células cancerosas escapar de los efectos supresores del TGF-β para entender la progresión tumoral y diseñar terapias que permitan bloquear los efectos pro-tumorogénicos del TGF-β en el carcinoma hepatocelular.

Los resultados presentados demuestran que los hepatocitos neonatales murinos (MH), al exponerlos de manera crónica al TGF-β, se muestran resistentes a sus efecto supresor tumoral y sufren EMT (Figuras 1,2) tal y como hemos descrito en hepatocitos fetales (Valdes et al. 2002) y células de hepatoma de rata (Caja et al. 2007). Utilizamos este modelo debido a que los hepatocitos adultos no transformados son incapaces de inducir Snail y promover EMT, probablemente debido a su incapacidad para activar el EGFR (Caja et al. 2007) y a la ausencia de Ras activado, que se ha mostrado crucial en la EMT inducida por TGF-β en otras células (Horiguchi et al. 2009, Grande et al. 2009, Peinado et al. 2003, Gotzmann et al. 2002). De manera interesante, la sobreexpresión de Snail1 es suficiente para inducir EMT en hepatocitos adultos no transformados (Franco et al. 2010). De esta manera mostramos que Snail1 juega un papel muy importante en la regulación de esta transición, ya que su cancelación impide la pérdida de Cadherina E y la EMT mediada por TGF-β (Figura 4).

Snail1 también es importante en la respuesta apoptótica mediada por TGF-β, ya que su cancelación aumenta esta respuesta en células MH (Figura 5), lo que indica que Snail1 supera la muerte celular inducida por TGF-β. En acuerdo con nuestros resultados, se ha publicado que Snail1 promueve resistencia a apoptosis en asociación con estimulación de Akt y represión de PTEN, a través de la unión de Akt2 al promotor de Cadherina E (Villagrasa et al. 2011). A la inversa, la activación de Snail confiere completa resistencia a muerte en células MH y en hepatocitos adultos obtenidos de ratón transgénico Snail1-ER tanto en respuesta al TGF-β (Franco et al. 2010) como su familia es capaz de conferir resistencia a muerte por retirada de factores de supervivencia o estímulos apoptóticos entre otros (Inoue et al. 2002, Perez-Losada et al. 2003, Vega et al. 2004, Kajita et al. 2004, Vitali et al. 2008, Martinez-Alvarez et al. 2004).

No hay dudas que la vía PI3K interfiere con los efectos apoptóticos del TGF-β en diferentes tipos celulares (Chen et al. 1998) e incluso el TGF-β induce señales anti-apoptóticas mediante la activación de Akt (Song et al. 2006, Wilkes et al. 2005, Valdes et al. 2004) y media carcinogénesis a través de la transactivación del EGFR en células de hepatoma (Caja et al. 2007). Es también conocida que la activación temprana de Akt está relacionada con la capacidad del TGF-β de transactivar las vías de c-Src y EGFR (Park et al. 2004, Murillo et al. 2005). En nuestro modelo de células MH demostramos en la Figura 6 que los ligandos del EGFR están implicados en la supervivencia mediada por Snail1, ya que su cancelación impide la regulación de éstos por TGF-β. Este resultado correlaciona con la inhicición de la activación de Akt, Erks y familia c-Src por TGF-β tras la cancelación de Snail (Figura 6), otorgándole a éste un papel importante en las señales de supervivencia mediadas por TGF-β en hepatocitos.

Existen evidencias que muestran que la sensibilidad a la apoptosis inducida por el TGF-β es regulada por la relación entre Akt/PKB y Smads y el ratio de proteína Smad3 sobre los niveles de Akt/PKB define si una célula epitelial sufre apoptosis en respuesta al TGF-β (Conery et al. 2004, Remy et al. 2004, Song et al. 2006, Kato et al. 2009). La alteración de este balance afecta a la respuesta apoptótica pero no a la inhibidora del crecimiento mediada por TGF-β (Conery et al. 2004). En nuestro modelo de células MH, la fosforilación de Smad3 se adelanta al cancelar Snail1 (Figura 7). Smad2, que se ha implicado en inhibición del crecimiento en hepatocitos y es necesaria para el mantenimiento de un fenotipo epitelial estable en hepatocitos primarios *in vitro* (Ju et al. 2006) muestra una fosforilación similar independiente del silenciamiento o no de Snail (Figura 7).

Previamente hemos publicado que el TGF-β induce señales anti-apoptóticas en hepatocitos fetales (Murillo et al. 2005), células de hepatoma (Caja et al. 2007) y células tumorales hepáticas humanas (Caja et al. 2009), mediante el aumento de las proteínas anti-apoptóticas de la familia Bcl-2 Bcl-xL y Mcl-1. También se ha demostrado la importancia de Bcl-xL, Mcl-1 así como de Bim y Bmf en hepatocitos y otros modelos celulares (Ramjaun et al. 2007, Romano et al. 2008). Bim y Bmf son activadores de las proteínas Bax y Bak (Wildey et al. 2003), que son los miembros pro-apoptóticos efectores del programa de muerte mediada por mitocondria. Su importancia en apoptosis ha sido probada en diferentes modelos celulares sistemas (Karbowski et al. 2006) (Ling et al. 2008). Nuestros resultados indican que la cancelación de Snail en células MH produce un aumento e inducción temprana de los niveles de Bim y Bmf, una reducción en la expresión de Bcl-xL y Mcl-1 así como, consecuentemente, un aumento en el porcentaje de células expresando una conformación activa de Bax o Bak (Figura 8). Estos resultados indican la importancia de Snail1 en la regulación del balance entre miembros pro-apoptóticos versus anti-apoptóticos de la familia Bcl-2 y un papel importante en la prevención de la apoptosis mitocondrial mediada por TGF-β en hepatocitos.

Un aspecto importante en el proceso metastático es la capacidad de sobrevivir a la muerte por falta de anclaje a un sustrato (anoikis) en la sangre o el sistema linfático (Chambers et al. 2002), ya que esta independencia de sustrato favorece la expansión e invasión de tejidos adyacentes y la diseminación. Existen diversas estrategias en las células tumorales para superar la anoikis como la hiperactivación de vías proliferativas (Zhao et al. 2009) o la implicación de reguladores de la EMT como β-catenina (Fischer et al. 2007), Slug (Leong et al. 2007), NF-κB, Snail o Twist en estas señales de supervivencia (Guadamillas et al. 2011). También se han implicado proteínas de la familia Bcl-2 en este proceso como Bax (Owens et al. 2009) o Bim (Reginato et al. 2003), que se relacionan directamente con el aumento de Bim y Bax debidos a la cancelación de Snail (Figura 8) que llevan a una menor resistencia a anoikis en nuestro modelo (Figura 9). Así Snail también controla la adhesión de los hepatocitos y confiere resistencia a la apoptosis inducida por pérdida de contacto (como la muerte por anoikis), apoyando datos previos que implican a Snail en la regulación de la adhesión de la célula a la matriz extracelular (Haraguchi et al. 2008) y un papel en el eje Twist-Snail en la EMT y resistencia a anoikis inducida por TrkB en células epiteliales intestinales (Smit et al. 2009).

Como consecuencia de la incapacidad de las células tumorales de responder a los efectos pro-apoptóticos del TGF-β y su habilidad para responder a la inducción de EMT, las células cancerígenas hepáticas superan los efectos supresores tumorales del TGF-β. Nuestros resultados muestran que la cancelación de Snail1 en dos líneas de carcinoma hepatocelular humano (HCC), Hep3B y SK-Hep1, restablece la capacidad de las células de sufrir apoptosis, el papel más importante del TGF-β en el control de la progresión del carcinoma hepatocelular (Figuras 10,11). De hecho la función supresora tumoral del TGF-β mediada por Smad3 es a través de la represión de Bcl-2 (Yang et al. 2006b) y Smad3 también es necesaria para la inducción de Snail por TGF-β (Sato et al. 2003), que actúa de manera opuesta incrementando Bcl-xL y Mcl-1. Así que quizás la progresión del carcinoma hepatocelular puede depender del equilibrio entre la activación de dos dianas de Smad3 como son Bcl-2 y Snail.

SPARC ha sido claramente implicado en el desarrollo tumoral aunque su papel es controvertido. Así existe sobreexpresión de SPARC en varios tumores malignos epiteliales (Clark and Sage 2008), promueve metástasis (Minn et al. 2005) y su expresión en melanoma correlaciona con agresividad y mal pronóstico (Massi et al. 1999). Recientemente se ha relacionado SPARC en la adquisición de rasgos mesenquimáticos que contribuyen a la diseminación del melanoma (Girotti et al. 2011). El papel de SPARC en HCC, cirrosis, fibrosis o hígado normal es controvertido. Por una parte está altamente expresado en fibrosis en comparación al hígado sano (Frizell et al. 1995) y su cancelación atenúa la fibrosis (Camino et al. 2008, Atorrasagasti et al. 2011). Asimismo, sus niveles se encuentran elevados en hepatocarcinoma en comparación con el tejido no tumoral (Luo et al. 2006) e indica susceptibilidad a HCC (Segat et al. 2009). Por otra parte, se sugiere que la sobreexpresión de SPARC en líneas de HCC (HepG2,

Hep3B y Huh7) conduce a una reducción de la tumorigenicidad mediante la inducción de MET (Atorrasagasti et al. 2010). En nuestros modelos celulares hepáticos, sólo la línea tumoral FaO de hepatoma pero no los MH son capacees de inducir la expresión de SPARC en la EMT inducida por TGF-β (Figuras 1,12), lo cual asocia la inducción de SPARC por TGF-β a malignidad hepática. Como se demuestra en la Figura 14, la cancelación de SPARC reduce la EMT inducida por TGF-β en células FaO, en acuerdo con la capacidad de SPARC de adquirir un fenotipo mesenquimático y capacidad migratoria en melanoma (Robert et al. 2006, Smit et al. 2007, Girotti et al. 2011) y la asociación entre SPARC y EMT en diversos tipos celulares (Vered et al. 2010, Conant et al. 2011) (Castilla et al. 2011) o el hecho de que la EMT inducida por Snail1 también está asociada a una fuerte inducción de SPARC (Moreno-Bueno et al. 2009, Moreno-Bueno et al. 2006).

Aunque se ha sugerido que Snail1 regula de manera directa la expresión de SPARC en melanoma (Kuphal et al. 2005), no encontramos cambios en los niveles de SPARC tras la cancelación de Snail1 en células FaO (resultados no mostrados). En cambio, encontramos una reducción en la expresión de Snail1 al cancelar SPARC (Figura 16). Esto puede ser debido a la comunicación entre TGF-β y SPARC como se ha demostrado en otros sistemas (Francki et al. 1999, Bassuk et al. 2000, Francki et al. 2004). De hecho se ha propuesto un bucle regulador autocrino y recíproco entre SPARC y la señalización por TGF-β1 en células epiteliales mamarias (Schiemann et al. 2003) y estamos investigando si la cancelación de SPARC afecta a la señalización por TGF-β en células FaO. De hecho, hemos publicado recientemente que la inducción de Snail1 en FaO y células MH requiere de la activación del TβRI (receptor I del TGF-β) y NF-κB (Franco et al. 2010), en acuerdo con Brancl y colaboradores que han demostrado que la vía TGFβ–IKKα–Smad induce la transcripción de Snai1 y Snai2 (Brandl et al. 2010).

El papel de SPARC en las funciones supresoras del TGF-β en células FaO se muestra en la Figura 15. Es más, describimos que la depleción de SPARC induce parada del ciclo celular en fase G_0/G_1 y hace a las células más sensibles a los efectos pro-apoptóticos del TGF-β, incrementando la apoptosis mitocondrial en células FaO (Figura 15). Esta nueva función de SPARC sugiere que su expresión puede ser importante en la EMT dirigida por TGF-β, manteniendo la supervivencia celular y facilitando el proceso invasivo.

El papel de SPARC en supervivencia fue descrito cuando se identificó como un gen de respuesta a estrés (Sage et al. 1986). En acuerdo a esto, ha sido recientemente implicado en la apoptosis inducida por estrés (Weaver et al. 2008, Chang et al. 2010) y se ha demostrado que promueve supervivencia celular en respuesta a estrés en células de melanoma a través de la fosforilación de Akt y la degradación de p53 (Fenouille et al. 2011). Estamos investigando la implicación de esta vía en nuestro sistema celular FaO.

Estos resultados apuntan a un papel para Snail y SPARC en contrarrestar los efectos supresores tumorales del TGF-β en hepatocitos, células de hepatoma y células de cáncer hepático, cambiando la respuesta hacia la promoción tumoral, haciéndolas

resistentes a la muerte celular y promoviendo la EMT y adquisición de propiedades invasivas.

Resultados de nuestro grupo muestran que el tratamiento crónico con TGF-β de células FaO selecciona a una subpoblación que sufre EMT, llamada TβT-FaO (Figura 17) que muestran un fenotipo mesenquimático, más migratorio, desdiferenciado y son resistente a los efectos supresores del TGF-β (Bertran et al. 2009). Inoculamos las células FaO y TβT-FaO de manera subcutánea e intrasplénica en ratones Balb/c desnudos para explorar su potencial tumorogénico.

La primera aproximación nos permite monitorizar y analizar la aparición de tumores, observando similares tiempo de duplicación tumoral y latencia (Figura 18). A pesar de que no encontramos diferencias en la aproximación subcutánea, existe una reducción en el ratio de proliferación (marcaje por KI-67), un alto marcaje para p-Smad2 (Figura 20) y un ligero retraso en la máxima incidencia tumoral (Tabla I) en los tumores inducidos por las células TβT-FaO en comparación con las células FaO, sugiriendo que el tratamiento de TGF-β *in vitro* ha afectado de algún modo la progresión del ciclo celular tumoral.

Los tumores obtenidos por la inyección intrasplénica de células FaO (Figura 18) recuerdan histológicamente a un HCC: células tumorales atípicas con abundante citoplasma eosinofílico y poco estroma en un área parte de éstas proliferando para reemplazar las células hepáticas normales (Komuta et al. 2008). Así estos tumores presentan múltiples focos rodeados de tejido conectivo por toda el área tumoral (Figura 19). En cambio, los tumores obtenidos a partir de las células TβT-FaO (Figura 18), muestran hepatocitos displásicos con un núcleo ampliado (alto ratio núcleo:citoplasma) que apunta a un comportamiento agresivo, poca supervivencia supervivencia y características de célula progenitora como se ha descrito anteriormente (Carvalho et al. 1997, Zhou et al. 2004b, Malehmir et al. 2012). Estos tumores muestran lesiones heterogéneas mayoritariamente unifocales y mayores que los inducidos por células FaO. La mayoría son muy irrigadas y parecen menos diferenciadas recordando a un hepatoblastoma, HB, (llamadas TβT-R). La capsula tumoral de los hepatoblastomas normalmente contiene abundantes vasos sanguíneos con zonas centrales necróticas (Benesch et al. 1998) y de manera interesante algunos animales injectados con células TβT-FaO presentan una anemia severa. Las lesiones TβT-R no tienen una estructura organizada como los otros tumores observados (Figura 19) y presentan múltiples sinusoides como se ha descrito anteriomente (Sakairi et al. 2001a). La inyección de células TβT-FaO también induce la aparición de lesiones blanquecinas que recuerdan un HCC o un colangiocarcinoma (CC), llamadas TβT-W (Figura 19). Se consideran rasgos típicos de CC las áreas tumorales pequeñas de formación papilar o glandular con producción de mucinas y abundante estroma fibroso (Komuta et al. 2008) y los tumores moderadamente diferenciados o poco diferenciados muestran patrones glandulares o tubulares con un patrón o formación de estructuras similares a cordones, con abundante pleomorfismo celular y estructuras distorsionadas en los poco diferenciados

(Nakanuma et al. 2003). La lesión TβT-W presenta abundantes áreas nodulares con células polarizadas en círculo, que recuerdan estructuras ductales siguiendo un patrón en cordón (Figura 19).

La evaluación de la velocidad de proliferación celular a través del marcaje de KI-67 muestra que es mayor en las lesiones FaO>TβT-W>TβT-D (Figura 20) que se correlaciona con un crecimiento más veloz de los cultivos obtenidos mediante tinción con Cristal Violeta (Figura 23) y con la baja proliferación de los hepatoblastomas (Zimmermann 2005). La evaluación de la apoptosis tardía mediante TUNEL muestra que los tumores obtenidos de las células TβT-FaO no muestran apenas células apoptóticas (Figura 20) y el único cultivo que induce actividad caspasa-3 es el FaO-D, correspondiente a la lesión FaO (Figura 24), en acuerdo con resultados previos que indican que las células tratadas crónicamente con TGF-β son resistentes a la apoptosis inducida por éste (Valdes et al. 2002, Bertran et al. 2009). La baja velocidad de crecimiento de los tumores TβT-FaO respecto a los FaO, conjuntamente con la resistencia a la apoptosis, explica la velocidad crecimiento similar de los tumores, así como el parecido marcaje por TUNEL y KI-67 en los tumores FaO indica una proliferación compensatoria de los hepatocitos.

El análisis del estado de activación de Smad2 muestra una señal débil nuclear en la lesión FaO<TβT-W<TβT-D (Figura 20). Se ha descrito marcaje de p-Smad2 nuclear en estadios tardíos de HCC (Wu et al. 2007) y hepatoblastomas (Hua et al. 2005). El hecho de una alta activación de la señalización por TGF-β, juntamente con la resistencia a apoptosis, puede indicar que las lesiones TβT-FaO presentan un estadio avanzado de tumorogénesis hepática.

Respecto a marcadores de EMT, las lesiones FaO muestran un marcaje fuerte y bien estructurado de Cadherina E, aunque también hay regiones sin marcaje y con una expresión débil de Vimentina (Figura 21), sugiriendo un proceso de EMT *in vivo*. Existe una correlación entre una baja expresión de Cadherina E, el grado tumoral y metástasis en HCC (Asayama et al. 2002, Zhai et al. 2008, Fransvea et al. 2008, van Zijl et al. 2009) y se ha sugerido la idea de una EMT *in vivo* en HCC a través de la proteína de matriz extracelular Laminina-5 (Giannelli et al. 2005). Por otra parte, la lesión TβT-W muestra una expresión de Cadherina E bien organizada y con expresión fuerte en las estructuras ductales parecidas a colangiocitos y conservan una expresión media de Vimentina en otras áreas (Figura 21). Este cambio de expresión respecto a las células TβT-FaO parentales puede indicar que tras la EMT *in vitro* las células han sufrido un proceso de transdiferenciación *in vivo* parecido al proceso MET, que les ha permitido adquirir un fenotipo tipo colangiocito a pesar de proceder de un hepatoma. Este hecho puede explicarse porque anteriormente hemos indicado que el proceso de EMT confiere características de progenitores hepáticos (del Castillo et al. 2008, Caja et al. 2011a) y de célula progenitora en otros modelos (Al-Hajj et al. 2003, Liu et al. 2007a, Honeth et al. 2008) y las células TβT-FaO han actuado como tales, transdiferenciando a una líneas colangiocítica. En cambio, la lesión TβT-R muestra un marcaje décil para Cadherina E y

fuerte para Vimentina (Figura 21) como las células TβT-FaO parentales y que se corrobora en la caracterización fenotípica de los cultivos derivados de los tumores (Figuras 25,27). Los hepatoblastomas acostumbran a perder el marcaje por Cadherina E (Anna et al. 2003, von Schweinitz et al. 1996) debido a mutaciones en el gen de la β-catenina (Takayasu et al. 2001, Park et al. 2001, Anna et al. 2000). No hemos evaluado aún los niveles de β-catenina, pero estos tumores muestran altos niveles de Snai1, Snai2, Vimentina y SPARC (Figuras 24,27), característicos de un proceso de EMT.

En resumen, los tumores derivados de las células TβT-FaO pueden tanto conservar el fenotipo mesenquimático diferenciado, en cuyo caso se forma un hepatoblastoma, o transdiferenciar a otro tipo tumoral, como es el caso de las lesiones colangiocíticas TβT-W, reflejando la enorme plasticidad de las células tumorales hepáticas.

Conectando con esta idea, los tumores FaO expresan, como las células FaO (Bertran et al. 2009), Citoqueratina (CK) 18 pero no CK7 o CK19, que son típicos en hepatocitos de-diferenciados o en colangiocitos diferenciados, y los tumores TβT-FaO expresan las tres CKs, con expresión de CK19 y CK18 y casi negativas para CK7 en el caso de la lesión TβT-W y con alta expresión de CK18 aunque también positiva para CK7 y CK19 en el caso de la lesión TβT-R (Figura 22). De acuerdo con estos resultados, la inoculación intrasplénica de hepatocitos tratados con TGF-β que han sufrido EMT genera lesiones con marcaje para CK19, sugiriendo un fenotipo más inmaduro (Zulehner et al. 2010). Un número importante de pacientes de HCC expresan marcadores de células biliares/progenitoras (Roskams 2006) y se ha sugerido que las células cancerígenas progenitoras circulantes sean la fuente de la recurrencia de los HCC (Fan et al. 2011).

Decidimos obtener tumores de las diferentes lesiones, llamados FaO-D, TβT-W-D y TβT-R-D, para analizar en profundidad los resultados obtenidos y afinar su caracterización. La morfología del cultivo FaO-D (Figura 23) es similar a las células FaO parentales tanto a nivel de cultivo (estructura en parénquima) como de distribución del citoesqueleto de actina de manera típicamente epitelial (Bertran et al. 2009) (Figura 25). La morfología del cultivo TβT-R-D es similar a las células TβT-FaO parentales, con apariencia fibroblastoide y células en forma de huso (Bertran et al. 2009) (Figuras 23,25). En cambio, el cultivo TβT-W-D presenta un fenotipo heterogéneo con células organizadas en parénquimas, que muestran una actina pericelular (Figura 25), y otras que muestran un fenotipo fibroblastoide, tal y como se describe la línea HepaRG derivada de un HCC y que sus autores relacionan con características de de hepatoblasto/células progenitoras (Guillouzo et al. 2007).

Estudiamos el estadio de diferenciación de los diferentes cultivos obtenidos respecto a las células parentales. El factor de transcripción HNF-4α (factor nuclear del hepatocito) es esencial para la diferenciación hepatocítica (Pontoglio et al. 1996, Li et al. 2000, Hayhurst et al. 2008) y su pérdida, aumento de la expresión de HNF1-β y de CK19 son características de la diferenciación hacia línea ductal (Limaye et al. 2010). Por otra parte, HNF4 muestra un patrón de expresión inverso al de CK19 en HCC (Andersen et al.

2010). Los cultivos TβT-FaO, TβT-W-D and TβT-R-D han perdido la expresión de HNF4α aunque mantienen la expresión de HNF3β (expresado en estadios iniciales de la diferenciación hepática), sugiriendo que ha ocurrido un proceso de transdiferenciación debido al tratamiento crónico con TGF-β (Figura 27). La expresión de CK19 en los cultivos derivados de TβT-FaO refuerza la idea de un paso atrás a un estadio anterior de diferenciación por el tratamiento in vitro con TGF-β, que puede permitir a las células diferenciarse tanto a hepatocitos como a colangiocitos. De manera interesante, EpCAM (molécula de adhesión a células epiteliales), un marcador expresado en colangiocitos y hepatoblastos (Tanaka et al. 2009, Okabe et al. 2009b, Cardinale et al. 2011) se encontró sólo en FaO-TβT, TβT-W-D and TβT-R-D (Figura 27). Otro marcador de célula progenitora también muy expresado en colangiocitos, el receptor c-kit (Crosby et al. 2001), se encuentra altamente expresado en el cultivo TβT-W-D (Figuras 27,28). c-kit es también un factor de pronóstico (Ernst et al. 1998, Tonary et al. 2000), se encuentra en un alto porcentaje de hepato-colangiocarcinoma combinados (Zhang et al. 2008) y se correlaciona con alta proliferación en hepatoblastoma (Sakairi et al. 2001a). En acuerdo con esto, el cultivo TβT-R-D (sin expresión de c-kit en la Figura 28) muestra menor velocidad de proliferación que el cultivo TβT-W-D (tinción por Cristal Violeta en la Figura 23 y tinción por KI-67 en la Figura 20). Las células progenitoras hepáticas (Yin et al. 2002, Fougere-Deschatrette et al. 2006) son capaces de formar estructuras ductales cuando se siembran en Matrigel y el único cultivo capaz de hacerlo es el TβT-W-D (Figura 28), confirmando su linaje colangiocítico.

TGF-β1 y FGF-b tienen un papel en el desarrollo y diferenciación del hígado (Zaret 2001, Lemaigre and Zaret 2004, Yoshida et al. 2007, del Castillo et al. 2008) y las vías TGF-β/BMP y Notch en la diferenciación ductal (Ader et al. 2006). Nuestros resultados sugieren la enorme plasticidad de los linajes de las células hepáticas, ya que el tratamiento crónico con TGF-β les confiere a unas células con fenotipo hepatocito la capacidad de transdiferenciarse a colangiocitos. En sintonía con estos resultados, Michalopoulos y colaboradores han mostrado que hepatocitos fetales pueden diferenciarse a células biliares *in vitro* mediante la incubación con EGF y/o HGF (Michalopoulos et al. 2001, Michalopoulos et al. 2002, Michalopoulos et al. 2005, Nishikawa et al. 2005, Limaye et al. 2008).

En resumen, la inoculación intrasplénica de células FaO de hepatoma de rata da lugar a tumores que parecen HCC mientras que el tratamiento crónico in vitro con TGF-β de estas células cambia su potencial tumorogénico. El crecimiento tumoral es similar, pero el fenotipo de las lesiones refleja un fenotipo de célula progenitora que provoca la aparición de tumores menos diferenciados (hepatoblastomas) o la transdiferenciación hacia una línea tumoral distinta (colangiocarcinomas). Esta heterogeneidad puede resultar por la renovación de las células del tumor, por adaptación al entorno o por la presencia de células cancerígenas progenitoras (Vermeulen et al. 2008, Bioulac-Sage et al. 2001). Estudios adicionales deben realizarse para clarificar el origen.

CONCLUSIONES

Primera. La cancelación específica de Snail1 en hepatocitos murinos y en células de hepatocarcinoma humano incrementa la apoptosis mitocondrial mediada por TGF-β, correlacionando con la inhibición de la expresión de los ligandos del receptor del factor de crecimiento epidérmico y la desregulación del balance entre miembros pro- y anti-apoptóticos de la familia Bcl-2.

Segunda. La cancelación específica de SPARC en células de hepatoma impide la regulación positiva de Snail1 e incrementa la apoptosis inducida por TGF-β.

Tercera. El tratamiento crónico in vitro con TGF-β de células FaO cambia su potencial tumorogénico. El crecimiento tumoral es similar, pero el fenotipo de las lesiones refleja un fenotipo de célula progenitora.

Cuarta. El cultivo primario de células de los tumores obtenidos tras la implantación intraesplénica de células de hepatoma tratadas con TGF-β revela la aparición de tumores menos diferenciados (hepatoblastomas) o la transdiferenciación a un linaje tumoral hepático diferente (colangiocarcinoma).

CONCLUSIÓN FINAL

Las células tumorales que superan los efectos supresores del TGF-β responden al mismoinduciendo la expresión de Snail1 y SPARC, los cuales promueven tanto EMT como supervivencia y pueden cambiar su potencial tumorogénico para provocar la aparición de tumores menos diferenciados o la transdiferenciación hacia un linaje tumoral hepático diferente

III. INTRODUCTION

1. Apoptosis

1.1. Main proteins involved in apoptosis: Caspases and Bcl-2 family

1.1.1. Caspases

The caspases (short for cysteine aspartase) are a multifunctional, highly regulated family of enzymes that catalyze a biologically diverse set of reactions like apoptosis and other cellular remodeling events.

Caspases lead the cell to completely different end points in spermatid individualization, macrophage differentiation, cornification, skeletal muscle differentiation, and erythropoiesis among other processes as revised by Crawford and collaborators (Crawford and Wells 2011). Apoptosis is a form of programmed cell death seen in metazoans, characterized by membrane blebbing, chromatin condensation, and other morphological features. It's a process tightly regulated and conserved throughout evolution and it's essential for the development and the maintenance of tissue homeostasis (Youle and Strasser 2008, Chowdhury et al. 2006).

As review in Olsson and collaborators, classification of human Caspases is based either on their function, the size of their pro-domain or cleavage specificity. Considering the first criteria, caspase-1, -4 and -5 belong to the group I (inflammatory) caspases that are involved in cytokine maturation. These proteases are primarily associated with the innate immunity against pathogens. Regulation of apoptosis, on the other hand, is controlled by group II caspases, which are divided into two classes: initiator (apical) caspases (caspase-2, -8, -9 and -10) and effector (executioner) caspases (caspase-3, -6 and 7). Like most proteases, caspases are synthesized as immature zymogens requiring specific proteolysis for activation (Khan and James 1998). Initiator caspases are activated upon recruitment to scaffolding complexes (Boatright and Salvesen 2003) and then go on to cleave and activate the downstream effector caspases. Effector caspases are constitutively produced in cells as dimers and proteolytic processing by an initiator enzyme is required to trigger their activity. Being active, effector caspases target a broad spectrum of cellular proteins, ultimately leading to cell death. In contrast to effector caspases, initiator caspases are translated as monomeric zymogens. Formation of multicomponent complexes triggers initiator caspase dimerization sufficient for their activation (Boatright et al. 2003). The death-inducing signaling complex (DISC), the apoptosome and the p53-induced protein with a death domain (PIDD)osome are protein assembly platforms that can recruit caspase-8/-10, -9 and -2, respectively (Olsson and Zhivotovsky 2011).

Thus the effector Caspase-3 is the one we measured its activity in order to define TGF-β apoptosis, here it's summarized its role in cancer as reviewed in Olsson and

collaborators. In a screen of primary breast tumor samples obtained from patients undergoing breast surgery, it was found that approximately 75% of the tumors as well as morphologically normal peritumoral tissue samples lacked caspase-3 transcripts and caspase-3 protein expression (Devarajan et al. 2002). In contrast, two reports describe upregulation of caspase-3 in clinical breast tumor samples (O 'Donovan et al. 2003, Nakopoulou et al. 2001). The explanation for this discrepancy may be due to the fact that the Caspase-3 gene can give rise to an alternative splicing variant known as caspase-3s, which has antiapoptotic function (Huang et al. 2001). Co-expression of caspase-3 and its splice variant has been found in diverse tumor cell lines, as well as in breast carcinomas, where the ratio of expression levels enabled identification of patients more likely to benefit from cyclophosphamide-containing chemotherapy (Vegran et al. 2006). At present, the role of caspase-3 in tumor formation/progression and tumor sensitivity to treatment is still unclear (Olsson and Zhivotovsky 2011).

1.1.2. Bcl-2 family

Currently, 15 Bcl-2 family proteins have been identified in mammals (Gross et al. 1999). All members of the Bcl-2 family share a close homology in up to four characteristic regions termed the BH (Bcl-2 homology) domains (BH1–4). They have either a pro-survival or a pro-apoptotic function (Danial 2007).

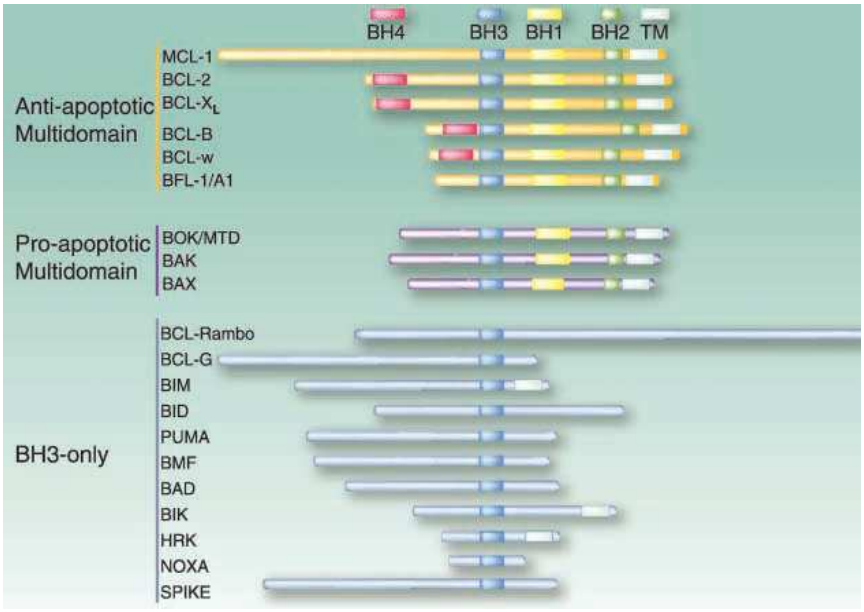


Figure i.1. Classification of BCL-2 family from Danial 2007.

The pro-survival proteins of the Bcl-2 family include Bcl-2, Bcl-w, Bcl-xL, Mcl-1 and A1. They possess up to four BH domains and all have similar 3D structures (Strasser 2005). The BH1, BH2 and BH3 domains interact to form a hydrophobic groove on the surface

of the protein. This groove accommodates the BH3 domain of its pro-apoptotic partners and is critical for the pro-survival function of these proteins. Overexpression of any of the pro-survival Bcl-2 family members prevents the death induced by many apoptotic stimuli, indicating a significant functional redundancy between these proteins. Under physiological conditions, however, it is rare that a single pro-survival protein ensures the survival of a cell population (Giam et al. 2009).

Bcl-2-regulated apoptosis requires Bax or Bak for mitochondrial damage. These two pro-apoptotic proteins contain BH1-3 domains, and have a 3D structure very similar to that of the pro-survival members of the family (Moldoveanu et al. 2006, Suzuki et al. 2000, Sattler et al. 1997). Mice lacking Bax show mild lymphoid hyperplasia and male sterility because of sperm-cell differentiation defects (Knudson et al. 1995). Mice lacking Bak have no documented defect so far. Most mice lacking both Bax and Bak complete embryonic development, but die around birth because they fail to nurse (Lindsten et al. 2000). Death induced by overexpression of pro-apoptotic BH3-only members of the Bcl-2 family seemed to be completely blocked in $Bax^{-/-}Bak^{-/-}$ cells, showing the strict requirement for Bax or Bak downstream of the BH3-only proteins. However, it is surprising that 10% $Bax^{-/-}Bak^{-/-}$ mice survived to adulthood and could live for several months (Lindsten et al. 2000).

Bax and Bak are the main effectors of the Bcl-2-regulated pathway. When activated, they permeabilize the mitochondria to allow the release of apoptogenic proteins into the cytosol. This is thought to occur through the formation of pores in the outer mitochondrial membrane. The pore theory appeared with the realization that the 3D structures of Bcl-xL (and later, of Bax) had significant similarity with the pore-forming translocation domain of the diphteria toxin (Suzuki et al. 2000). Despite intense efforts to show the existence of this pore, its molecular composition remains elusive. The presence of Bax or Bak is required to allow cytochrome c out of the mitochondria or large macromolecules out of synthetic lipidic vesicles, but how they achieve this not completely understood. Although Bax and Bak seem to have identical functions, one striking difference between them is their subcellular localization in healthy cells (Reed 2006). Bak is an integral membrane protein on the outer mitochondrial membrane, whereas inactive Bax is a largely cytosolic protein (Wolter et al. 1997). This is because the C-terminal membrane-anchoring domain of Bax is tucked into its hydrophobic pocket and an additional step is required to release it, allowing it to translocate to the mitochondria where it undergoes oligomerization (Nechushtan et al. 2001).

Bax and Bak proteins undergo a set of activation steps in response to apoptotic stimuli (Desagher and Martinou, 2000). Upon initiation of the apoptotic cascade, Bax translocated from cytoplasm to mitochondria, both Bax and Bak proteins change their conformation and form homo-oligomers (Suzuki et al. 2000). On the outer membrane of the mitochondria, oligomerized Bax and Bak may form a channel or membrane pore, which allows the release of cytochrome c and Smac/DIABLO (Annis et al. 2005).

During apoptosis, Bax translocates from the cytosol to the outer mitochondrial membrane (OMM), wherein it contributes to the formation of pores to release cytochrome-c. However, it remains unclear whether Bax translocation is sufficient to bring about MOMP (mitochondrial outer membrane permeabilisation) or whether Bax requires further signals on the OMM to be fully activated. It have previously shown that during mammary epithelial cell anoikis, Bax translocation does not commit cells to MOMP and detached cells are rescued if survival signals from the extracellular matrix (ECM) are restored. These findings implied that a second signal is required for mitochondrial Bax to fully activate and cause MOMP. Afterwards, it was identified that p38MAPK (mitogen-activated protein kinase) as this necessary signal to activate Bax after its translocation to mitochondria. The inhibition of p38MAPK did not prevent Bax translocation, but its activity was required for mitochondrial Bax to bring about MOMP. p38MAPK was activated and recruited to a high molecular weight mitochondrial complex after loss of ECM attachment. Artificially targeting p38MAPK to the OMM increased the kinetics of anoikis, supporting a requirement for its mitochondrial localization to regulate Bax activation and drive commitment to apoptosis (Owens et al. 2009).

The mammalian BH3-only proteins currently known include Bad, Bid, Bik/NBK, Bim/Bod, Bmf, Hrk/DP5, Noxa and Puma/BBC3. Depending on the definition of what constitutes a BH3 domain, several other proteins may also be included in this subfamily. The BH3-only proteins are pro-apoptotic and the only region that they share with each other and their relatives of the Bcl-2 family is the BH3 domain (Willis and Adams 2005). Overexpression of BH3-only proteins promotes apoptotic death in many cell types, but requires the presence of either Bax or Bak for the death to occur (Zong et al. 2001). This places them upstream of Bax/Bak in the chain of events that lead to caspase activation.

All BH3-only proteins bind strongly to at least some pro-survival members of the family. The BH3 domain of BH3-only proteins inserts in the hydrophobic groove formed by the BH1, BH2 and BH3 domains at the surface of the pro-survival molecules. Some BH3-only proteins (Bim, Puma and Bid) bind to all the prosurvival molecules, whereas others have a more limited repertoire (Chen et al. 2005). Bad, for example, binds Bcl-2, Bcl-xL and Bcl-w, but not Mcl-1 or A1. Noxa, by contrast, binds Mcl-1 and A1, but not the others. The BH3 domain seems to be entirely responsible for this interaction, and is absolutely required for the killing activity of the BH3-only proteins. In addition, Bim, Bid and Puma were also found to interact with Bax (Willis et al. 2007, Cartron et al. 2004, Marani et al. 2002). Bim, Bad and Bmf are unstructured in the absence of a binding partner, and only their BH3 domain becomes structured upon binding a pro-survival protein (Hinds et al. 2006). In contrast, Bid shows a structure strikingly similar to that of Bax and Bcl-xL, even though it lacks recognizable BH1 and BH2 domains (Chou et al. 1999, McDonnell et al. 1999).

1.2. Apoptosis extrinsic and intrinsic pathways

The two main apoptosis pathways are the extrinsic receptor-mediated and intrinsic mitochondrial pathways as revised in (Fulda and Debatin 2006).

1.2.1. Extrinsic pathway of apoptosis

Death receptors are members of the tumor necrosis factor (TNF) receptor gene superfamily that consists of more than 20 proteins with a broad range of biological functions, including regulation of cell death and survival, differentiation or immune regulation (Ashkenazi 2002, Walczak and Krammer 2000). Members of the TNF receptor family share similar, cysteine-rich extracellular domains. In addition, death receptors are defined by a cytoplasmic domain of about 80 amino acids called 'death domain', which plays a crucial role in transmitting the death signal from the cell's surface to intracellular signaling pathways. The best-characterized death receptors include CD95 (APO-1/Fas), TNF receptor 1 (TNFRI), TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1) and TRAIL-R2, whereas the role of DR3 (TRAMP/Apo-3/WSL-1/LARD) or DR6 has not exactly been defined (Walczak and Krammer 2000). The corresponding ligands of the TNF superfamily comprise death receptor ligands such as CD9 5 ligand (CD95L), TNFα, lymphotoxin-α (the later two bind to TNFRI), TRAIL and TWEAK, a ligand for DR3 (Walczak and Krammer 2000).

1.2.2. Intrinsic pathway of apoptosis

The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apoptosis inducing factor (AIF), Smac (second mitochondria derived activator of caspase)/DIABLO (direct inhibitor of apoptosis protein (IAP)-binding protein with low PI), Omi/HtrA2 or endonuclease G from the mitochondrial intermembrane space (Saelens et al. 2004, Cande et al. 2004). The release of cytochrome c into the cytosol triggers Caspase-3 activation through formation of the cytochrome c/Apaf-1/Caspase-9-containing apoptosome complex, whereas Smac/DIABLO and Omi/HtrA2 promote caspase activation through neutralizing the inhibitory effects to the IAPs (Inhibitor-Of-Apoptosis Protein) (Saelens et al. 2004).

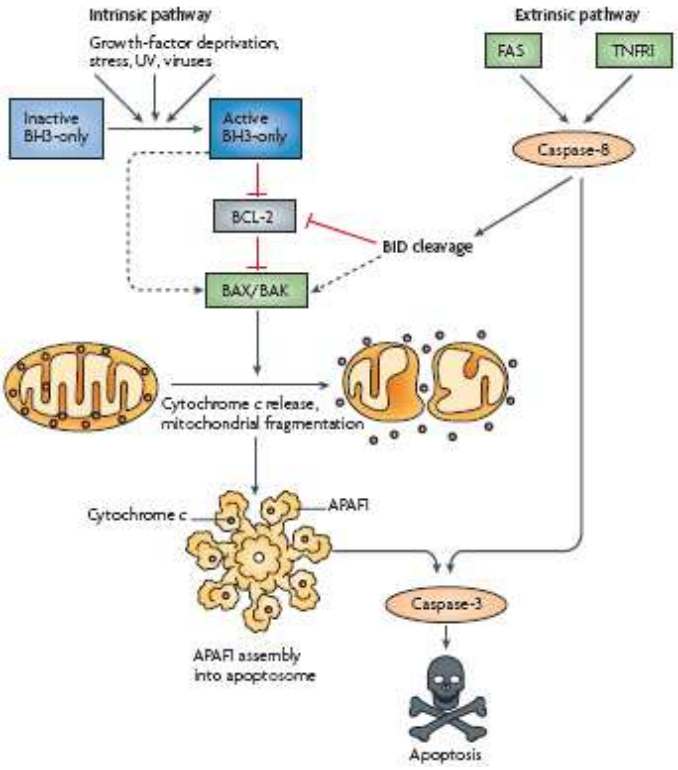


Figure i.2. Apoptosis pathways from Youle and Strasser 2008.

In the mitochondrial pathway of apoptosis, caspase activation is closely linked to permeabilization of the outer mitochondrial membrane by proapoptotic members of the Bcl-2 family (Green and Kroemer 2004). Numerous cytotoxic stimuli and proapoptotic signal transducing molecules converge on mitochondria to induce outer mitochondrial membrane permeabilization (Green and Kroemer 2004, Decaudin et al. 1998). This permeabilization is regulated by proteins from the Bcl-2 family, mitochondrial lipids, proteins that regulate bioenergetic metabolite flux and components of the permeability transition pore (Green and Kroemer 2004). Upon disruption of the outer mitochondrial membrane, a set of proteins normally found in the space between the inner and outer mitochondrial membranes is released, including cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G (Saelens et al. 2004). Once in the cytosol, these apoptogenic proteins trigger the execution of cell death by promoting caspase activation or by acting as caspase-independent death effectors (Saelens et al. 2004).

The release of cytochrome c from mitochondria directly triggers Caspase-3 activation through formation of the cytochrome c/Apaf-1/Caspase- 9-containing apoptosome complex (Cain et al. 2000). Once in the cytosol, cytochrome c binds to the C-terminal region of Apaf-1, a cytosolic protein with an N-terminal caspase-recruitment domain (CARD), a nucleotide-binding domain with homology to Caenorhabditis elegans CED-4

and a C-terminal domain containing 12–13 WD-40 repeats (Zou et al. 1997). Binding of cytochrome c to Apaf-1 facilitates the association of dATP with Apaf-1 and exposes its N-terminal CARD, which can now oligomerize and become a platform on which the initiator Caspase-9 is recruited and activated through a CARD–CARD interaction (Adrain et al. 1999). Consecutively, the executioner Caspase-3 is recruited to the apoptosome, where it is activated by the resident Caspase-9 (Bratton et al. 2001). Caspase-3 then cleaves key substrates in the cell to produce many of the cellular and biochemical events of apoptosis.

In certain cases, caspase activity instigated by cytosolic cytochrome c contributes to the drop of matrix metalloproteinase (MMP), as was shown by the use of synthetic caspase inhibitors and Apaf1$^{-/-}$ cells (Waterhouse et al. 2001). In addition, caspase activity further damages the function of permeabilized mitochondria by affecting the activity of complexes I and II, inevitably leading to the loss of MMP and the generation of reactive oxygen species (Ricci et al. 2004, Herrera et al. 2004, Carmona-Cuenca et al. 2006, Murillo et al. 2007, Ortiz et al. 2008, Sancho et al. 2009, Caja et al. 2009). Thus, secondary events resulting from cytosolic changes, caused by the release of cytochrome c and other mitochondrial proteins, can feed back on permeabilized mitochondria and affect their function. Importantly, this mitochondrial amplification loop of caspase activity may critically determine the response of cancer cells to cytotoxic treatments.

Other proteins released from mitochondria, such as Smac/DIABLO and Omi/HtrA2, facilitate caspase activation through neutralizing endogenous inhibitors of caspases, the inhibitor of apoptosis proteins (IAPs). Smac and its murine homolog DIABLO are nuclear encoded mitochondrial proteins, which contain a mitochondrial localization signal, which is proteolytically removed upon mitochondrial import to yield the mature 23 kDa protein (Du et al. 2000, Verhagen et al. 2000).

Although the release of cytochrome *c* into the cytosol directly triggers Caspase-3 activation through formation of the cytochrome *c*/Apaf-1/Caspase-9-containing apoptosome complex, Smac/DIABLO and Omi/HtrA2 indirectly promote caspase activation through antagonizing the inhibitory effects to IAPs (Saelens et al. 2004). Thus, a dynamic equilibrium exists between pro- and antiapoptotic effector molecules, which allow the cell to cope with limited mitochondrial damage, in which case IAPs can adequately block caspase activation initiated by a small amount of released cytochrome *c*. However, under circumstances where mitochondrial damage proceeds or simultaneously affects multiple mitochondria, the antiapoptotic hurdle imposed by IAPs can be overcome by the higher cytosolic concentration of their antagonists Smac/DIABLO and HtrA2/OMI, which neutralize IAPs by direct binding.

2. The Epithelial to Mesenchymal Transition (EMT)

2.1. EMT definition and physiological functions

Epithelial cells typically form sheets, tubes or vesicles, in which the cells establish an apical-basal polarity. Epithelial cells intimately associate with each other through laterally located, specialized cell-cell contact structures, i.e. tight junctions, adherens junctions and desmosomes (Chung and Andrew 2008). Tight junctions are membrane fusions at the lateral side close to the apical surface that provide intercellular sealing and protect against paracellular diffusion, and separate the distinct functions of the apical and basolateral surfaces (Tsukita et al. 2001). Occludin and claudins are important components of the intercellular tight junction strands, while the cytoplasmic components Zonula Occludens (ZO)-1, -2, -3 and p120 are integral to an undercoat structure. The cytoplasmic tails of ZO proteins attach to actin filaments, thus contributing to the strength and integrity of tight junctions (Tsukita et al. 2001). Adherens junctions are located adjacent to the tight junctions in the basolateral surface compartments of epithelial cells, and connect to cytoskeletal microfilaments. (Niessen and Gottardi 2008). The cytoplasmic domains of the transmembrane adhesion receptor E-cadherin bind tightly to β-catenin, a cytoplasmic protein that interacts with α-catenin, which in turn anchors to the actin cytoskeleton, either directly or indirectly via actin-binding proteins α-actinin and vinculin (Wheelock and Johnson 2003, Niessen and Gottardi 2008).

EMT is a transient and reversible switch from a polarized, epithelial, to a fibroblastoid or mesenchymal cellular phenotype, the latter exhibiting highly motile and invasive properties (Grunert et al. 2003, Thiery and Sleeman 2006). During EMT, the adherens junction complexes disassemble and the actin cytoskeleton reorganizes from an epithelial cortical alignment associated with cell-cell junctions into actin stress fibers that are anchored to focal adhesion complexes (Chung and Andrew 2008). Loss of E-cadherin is considered as a hallmark event of EMT that initiates a series of signaling events and major cytoskeletal reorganization. However, loss of E-cadherin should not be considered as the sole pivotal event in EMT, since blocking E-cadherin expression by transfection of antisense RNA does not induce a full EMT (Llorens et al. 1998). Furthermore, forced E-cadherin expression is insufficient to restore the epithelial phenotype in spindle carcinoma cells and does not reverse induced EMT (Navarro et al. 1993, Yang et al. 2004, Ohkubo and Ozawa 2004, De Craene et al. 2005, Ansieau et al. 2008).

Concomitant with the loss of epithelial cell-cell contact structures and actin reorganization, cells undergoing EMT acquire a mesenchymal identity. The mesenchymal phenotype is apparent from the expression of mesenchymal cytoskeletal proteins, such as Vimentin, and the increased deposition of extracellular matrix proteins, including collagens and fibronectin. These extracellular matrix components

stimulate integrin signaling and induce the formation of focal adhesion complexes, which facilitate cell migration (Miettinen et al. 1994, Imamichi and Menke 2007). The generally observed downregulation of E-cadherin also promotes the assembly of focal adhesion through activation of focal adhesion kinase (Frame and Inman 2008). Furthermore, decreased expression of E-cadherin during EMT is accompanied by increased expression of N-cadherin, which renders the cell more motile and invasive (Cavallaro and Christofori 2004, Deckers et al. 2006, Shirakihara et al. 2007). These different events result in a loss of apical-basal polarity that is critical to the maintenance of epithelial morphology as well as the function of the epithelial sheet. Furthermore, the loss of specialized cell-cell contact structures facilitates the increased migration, a defining behavioral property of cells that have undergone EMT (Grunert et al. 2003). With the loss of apical-basal polarity, cells acquire a front-back polarity that allows them to migrate in a directional fashion. The increased expression and activity of extracellular proteases, such as matrix metalloproteinases, allow the cells to degrade extracellular matrix proteins, thus allowing the migration to translate into an invasive behavior, whereby the cells delaminate and escape from their epithelial structures (Moustakas and Heldin 2007).

EMT can be triggered by different signalling molecules such as by epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), transforming growth factor β (TGF-β), bone morphogenetic proteins (BMPs), WNTs and Notch (Barrallo-Gimeno and Nieto 2005). Several families of transcription factors, including the Snail family, ZEB family and basic helix-loop-helix (bHLH) family, act in concert to control the EMT process and the changes in gene expression patterns that underlie EMT (Peinado et al. 2007, Moreno-Bueno et al. 2008b). These changes in the transcription program are complemented by non-transcriptional changes that help define the changes in cytoskeletal organization and cell shape and the interaction of the cell with its environment. EMT can be induced or regulated by various growth and differentiation factors, including TGF-β, growth factors that act through receptor tyrosine kinases, such as fibroblast growth factor, hepatic growth factor and platelet derived growth factor (PDGF), and Wnt and Notch proteins (Moustakas and Heldin 2007). Commonly used molecular markers for EMT include increased expression of the previously mentioned transcription factors, reduced expression of Cytokeratins, increased expression of Vimentin, S100A4 and N-cadherin, and nuclear localization of β-catenin (Lee et al. 2006b).

2.2. EMT physiological functions

Cells that have undergone EMT are endowed with the ability to transmigrate basement membranes and stromal tissues as well as to intravasate and pass the circulatory system. In general, this process is a central event in both early stages of embryonic development and in pathophysiological situations such as wound healing, chronic

inflammation and carcinoma progression as revised by van Zijl and collaborators (van Zijl et al. 2009).

There have been described three types of EMT (Kalluri and Weinberg 2009). EMTs type 1 are the ones associated with implantation, embryo formation, and organ development occur during a self-contained process with an implicit need to generate diverse cell types that share mesenchymal phenotypes and biomarkers. This EMT causes neither fibrosis nor uncontrolled systemic invasion by high-grade epithelial cancer cells; rather, it generates cells with a mesenchymal phenotype to create new tissue(s) with diverse functions. During early embryonic development, epithelial cell sheets convert into mesenchymal cells which is the basis for the formation of a three-layered embryo, a process known as gastrulation (Thiery 2002, Thiery and Sleeman 2006). In mammals, EMT takes place at the blastula stage during parietal endoderm formation, where epithelial precursors of the visceral endoderm get in contact with the mural trophectoderm in the marginal zone and generate newly mesodermal mesenchymal cells which later contribute to the extraembryonic tissues. Moreover, EMT also shows fundamental implications in organogenesis during formation of the heart, the musculoskeletal system, craniofacial structures and the peripheral nervous system.

EMTs type 2 are the ones associated with wound healing, tissue regeneration, and organ fibrosis (Kalluri and Weinberg 2009, Zeisberg and Neilson 2009). During wound healing, where the response to injury reduces the adhesiveness of epithelial cells in the epidermis through complex cellular and molecular interactions, EMT mechanisms increase the migratory phenotype in order to re-generate intact epithelial sheets (Bissell and Radisky 2001). 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. Presumably tissue regeneration is associated with inflammation and ceases once repair is achieved and inflammation is attenuated. In the setting of organ fibrosis, type 2 EMT can continue to respond to ongoing inflammation, leading eventually to organ destruction (Kalluri 2009). Organ fibrosis is mediated by inflammatory cells and fibroblasts as well as components of a complex ECM (Extracellular matrix). EMTs are found to be associated with fibrosis occurring in kidney, liver, lung, and intestine (Kim et al. 2006, Zeisberg et al. 2007a, Zeisberg et al. 2007b, Potenta et al. 2008). Inflammatory injury to the mouse kidney can result in the recruitment of a diverse array of cells that can trigger an EMT through their release of growth factors, such as TGF-β, PDGF, EGF, and FGF-2 (Fibroblast Growth Factor-2) (Strutz et al. 2002). The significance of TGF-β–induced EMT for progression of organ fibrosis has been demonstrated in studies using BMP-7, an antagonist of TGF-β signaling, in mouse models of kidney, liver, billiard tract, lung, and intestinal fibrosis (Zeisberg et al. 2003b, Zeisberg et al. 2003a). BMP-7 functions as an endogenous inhibitor of TGF-β–induced EMT (Zeisberg et al. 2003b, Zeisberg et al. 2003a). Chronic inflammation and fibrosis with increased TGF-β expression promotes hepatocytes to

change their phenotype and functions in order to escape apoptosis which is the basis for hepatocarcinogenesis. In this respect, studies on fetal rat hepatocytes that have undergone EMT upon TGF-β treatment have demonstrated that EMT leads to the resistance against TGF-β-mediated apoptosis. Survival of fibroblastoid derivatives of hepatocytes might be accomplished by the induction of epidermal growth factor (EGF)-R- and Src-dependent activation of PI3K (Phosphoinositide-3-Kinase)/Akt as well as by high levels of Bcl-XL and Snail (Valdes et al. 2002, Murillo et al. 2005). Studies using fibrosis tissue from humans have also demonstrated EMT (Rastaldi et al. 2002) and in patients with Crohn disease, an EMT was demonstrated in areas of fibrosis in the colon (Bataille et al. 2008).

EMT Type 3 occurs in epithelial cancer cells that differ genetically and epigenetically from untransformed epithelial cells. These changes, which mainly affect oncogenes and tumor suppressor genes, conspire with the EMT regulatory circuitry to produce outcomes far different from those observed in the other two types of EMT. Cells generated by type 3 EMT may invade and metastasize via the circulation and thereby generate systemic manifestations of malignant cancer progression (Kalluri 2009). This third type of EMT is explained in the next chapter.

2.3. EMT and cancer progression

EMT has been recognized as a crucial step in the metastatic cascade (Massague 2008, Thiery et al. 2009, Polyak and Weinberg 2009). The presence of an EMT signature can be correlated with the activation of EMT inducers such as Snail, Zeb, or Twist factors and the loss of E-cadherin (Thiery et al. 2009). EMT also correlates with invasiveness, a poor prognosis, and the most aggressive types of cancer such as the basal-type breast carcinomas (Massague 2008, Sarrió et al. 2008).

The genetic and epigenetic alterations undergone by cancer cells during the course of primary tumor formation render them especially responsive to EMT-inducing heterotypic signals originating in the tumor-associated stroma. Oncogenes induce senescence, and recent studies suggest that cancer cell EMTs may also play a role in preventing senescence induced by oncogenes, thereby facilitating subsequent aggressive dissemination (Smit and Peeper 2008, Ansieau et al. 2008, Weinberg 2008). In the case of many carcinomas, EMT-inducing signals emanating from the tumor-associated stroma, notably HGF, EGF, PDGF and TGF-β. Noncoding microRNAs are also components of the cellular signaling circuitry that regulates the EMT program. For example, microRNA 200 (miR200) and miR205 inhibit the repressors of E-cadherin expression, ZEB1 and ZEB2, and thereby help in maintaining the epithelial cell phenotype (Park et al. 2008, Gregory et al. 2008). Activation of EMT programs is also facilitated by the disruption of cell-cell adherens junctions and the cell-ECM adhesions

mediated by integrins (Gupta et al. 2005, Yang et al. 2006a, Hartwell et al. 2006, Taki et al. 2006, Mani et al. 2007, Weinberg 2008, Yang and Weinberg 2008, Mani et al. 2008).

The EMT program depends on a series of intracellular signaling networks involving, among other signal transducing proteins, ERK (Extracellular Signal-Regulated Kinase), MAPK (Mitogen-Activated Protein Kinase), PI3K, Akt, Smads, RhoB, β-catenin, LEF (lymphoid enhancer binding factor), Ras, and c-Fos as well as cell surface proteins such as β4 integrins, α5β1 integrin, and αVβ6 integrin (Tse and Kalluri 2007). The small GTPase RhoA maintains epithelial tight junctions. TGFβ has been shown to cause Rho degradation in a manner dependent upon ligand-initiated effects of TβRII, which leads to the direct phosphorylation of Par6, which leads to the recruitment of the ubiquitin ligase Smurf1. Smurf1 then targets RhoA for degradation (Ozdamar et al. 2005). Independent of the genetic background of EMT, platelet-derived growth factor (PDGF)A ligand and both PDGF receptor (PDGF-R) α and β were found to be upregulated along with autocrine PDGF secretion (Gotzmann et al. 2006). Inhibition of PDGF signaling revealed decreased migration in vitro and efficient tumor suppression in vivo, indicating an essential role of PDGF in TGF-β-mediated EMT of neoplastic hepatocytes. Upregulation of PDGF/PDGF-R has been further reported through the collaboration of oncogenic Ras and interleukin-like EMT inducer (ILEI) during the fibroblastoid conversion of Ras-transformed hepatocytes (Lahsnig et al. 2009). In addition, ILEI –a downstream target of TGF-β during hepatocellular EMT– collaborates with oncogenic Ras to induce both the activation signal transducer and activator of transcription STAT3 (Signal Transducers And Activators Of Transcription-3) and the nuclear accumulation of β-catenin via PDGF/PDGF-R. Importantly, interference with PDGF-R signaling abolishes nuclear β-catenin translocation upon TGF-β signaling in vivo (Fischer et al. 2007). This finding is of particular relevance since about 33-67% of HCC (human hepatocellular carcinoma) cases display nuclear accumulation of β-catenin (Lee et al. 2006a).

TGF-β can induce EMT in hepatocytes (Valdes et al. 2004, Murillo et al. 2005, Fischer et al. 2005, del Castillo et al. 2006, Caja et al. 2007, Kojima et al. 2008, Zhang et al. 2009, Pan et al. 2009, Battaglia et al. 2009, Copple 2010, Kaimori et al. 2010, Wang et al. 2010, Caja et al. 2011a). TGF-β can induce an EMT in Ras transformed hepatocytes, mammary epithelial cells (via MAPK), and MDCK (Madin-Darby canine kidney) cells; at the same time, Ras-activated PI3K inhibits TGF-β–induced apoptosis to facilitate this transition (Oft et al. 1996, Lehmann et al. 2000, Gotzmann et al. 2002, Janda et al. 2002). In mouse models of skin carcinoma and human colon cancer, the absence of TGF-β receptor expression actually confers better prognosis (Cui et al. 1996, Watanabe et al. 2001).

The connection between loss of E-cadherin expression by cancer cells and passage through an EMT has been established by many studies (Edelman et al. 1983, Tepass et al. 2000). For example, induction of the c-Fos oncogene in normal mouse mammary epithelial cell lines induces an EMT and is associated with a decrease in E-cadherin expression (Eger et al. 2000). β-catenin accumulation in the nucleus, which is often

associated with loss of E-cadherin expression, correlates with susceptibility to enter into an EMT and acquisition of an invasive phenotype (Kim et al. 2002b, Thiery 2002). Some studies have demonstrated that the epigenetic control of E-cadherin and β-catenin/LEF activity is important in establishing the metastatic potential of cancer cells (Birchmeier and Behrens 1994, Muta et al. 1996, Saito et al. 1999). Cell lines that lack E-cadherin show increased tumorigenicity and metastasis when transferred into immunodeficient mice (Birchmeier and Behrens 1994). Mutations in the E-cadherin gene have been identified in cancer cells, making them more susceptible to EMT and metastasis (Muta et al. 1996, Saito et al. 1999).

Many mouse studies and cell culture experiments have demonstrated that carcinoma cells can acquire a mesenchymal phenotype and express mesenchymal markers such as α-SMA (α-Smooth Muscle Actin), FSP1 (Fibroblast-specific protein 1), Vimentin, and desmin (Yang and Weinberg 2008). These cells typically are seen at the invasive front of primary tumors and are considered to be the cells that eventually enter into subsequent steps of the invasion-metastasis cascade, like intravasation, transport through the circulation, extravasation, formation of micrometastases, and ultimately colonization: the growth of small colonies into macroscopic metastases (Brabletz et al. 2001, Thiery 2002, Fidler and Poste 2008).

Breast tumor cells that undergo collective migration are capable of invading lymphatic vessels, whereas individually migrating cells can invade both lymphatic and blood vessels. Therefore, only individual cells seem to be able to disseminate through the bloodstream. Interestingly, these single cells show transient TGF-β signaling, a potent signal for the EMT (Giampieri et al. 2009). Interestingly, individual cells migrating from primary tumors in a model of breast carcinoma in mice have been isolated and characterized. In agreement with the previously proposed properties imposed on cells upon undergoing the EMT (Vega et al. 2004), the invasive cancer cells have an attenuated proliferation rate and are resistant to cell death in addition to expressing EMT markers (Roussos et al. 2010) and previous results from our group (Sanchez et al. 1999, Valdes et al. 2002, Valdes et al. 2004, Murillo et al. 2005, del Castillo et al. 2006, Caja et al. 2007, Bertran et al. 2009).

An apparent paradox comes from the observation that the EMT-derived migratory cancer cells typically establish secondary colonies at distant sites that resemble, at the histopathological level, the primary tumor from which they arose; accordingly, they no longer exhibit the mesenchymal phenotypes ascribed to metastasizing carcinoma cells. Reconciling this behavior with the proposed role of EMT as a facilitator of metastatic dissemination requires the additional notion that metastasizing cancer cells must shed their mesenchymal phenotype via a MET (Mesenchymal–Epithelial Transition) during the course of secondary tumor formation (Zeisberg et al. 2005). The tendency of disseminated cancer cells to undergo MET likely reflects the local microenvironments that they encounter after extravasation into the parenchyma of a distant organ, quite possibly the absence of the heterotypic signals they experienced in the primary tumor

that were responsible for inducing the EMT in the first place (Thiery 2002, Jechlinger et al. 2002, Bissell et al. 2002). These considerations indicate that induction of an EMT is likely to be a centrally important mechanism for the progression of carcinomas to a metastatic stage and implicates MET during the subsequent colonization process

2.4. EMT and stemness

It appears that signaling pathways involved in the regulation of stem cell function and niche-stem cell interactions can play some part in triggering EMT programs, potentially connected with the role of these programs in establishing and maintaining stem cell-like characteristics (Polyak and Weinberg 2009).

Recent data also implicate a role for TGF-β in regulating breast cancer stem cell phenotypes (Mani et al. 2008, Morel et al. 2008), demonstrating than the EMT process leads to the generation of breast cancer cells with stem cell-like characteristics as we previously published in rat hepatocytes (del Castillo et al. 2008) and human liver cells (Caja et al. 2011a). One group studying EMT analyzed the expression of EMT-associated genes in CD44+CD24– cells isolated from normal human breast tissue and from primary human breast carcinomas and established that most of the EMT-inducing transcription factors (Twist1, Foxc2, Snai1, Zeb2 (also known as Sip1) and Twist2), as well as Vimentin and Fibronectin, were expressed at far higher levels in CD44+CD24– stem cell-like cells than in more differentiated epithelial CD44–CD24+ cells (Mani et al. 2008).

Further analysis of Wnt/β-catenin signaling in the liver showed that nuclear β-catenin in neoplastic hepatocytes correlates with their transdifferentiation to early hepatocyte progenitors that abundantly express M2-pyruvate kinase (M2-PK) and Cytokeratin 19, but lack epithelial Cytokeratins 8 and 18. On the contrary, ablation of β-catenin by the overexpression of axin caused strong expression of Cytokeratins 8 and 18 (Zulehner et al. 2010). These data demonstrate that the TGF-β-dependent, nuclear accumulation of β-catenin results in a loss of epithelial markers and increased expression of hepatic progenitor markers. In agreement with these results, TGF-β provokes MMH (Met murine hepatocytes) epithelial cells to act as bipotential precursor cells concomitant with down-regulation of liver-specific gene expression (Spagnoli et al. 2000). Furthermore, TGF-β also induces the self-renewal capacity of patient-derived gliomas-initiating cells and prevents cell differentiation through the Smad-dependent induction of LIF (Leukemia inhibitory factor) (Penuelas et al. 2009). It's also been demonstrated the essential role of TGF-β in maintaining the pluripotency of human embryonic stem cells thought Smad2/3 signaling and in the maintenance of the inner cell mass from which stem cells are derived (James et al. 2005). Operating through an alternative mechanism, the hypomethylation of genes specifying transcription factors that program stem cell phenotypes may lead to EMTs and, in cancer cells, correlate with poorly differentiated cell phenotypes and increased risk of distant metastasis

(Bloushtain-Qimron et al. 2008). In addition, individual members of a group of six to eight transcription factors, as reviewed in (Polyak and Weinberg 2009), have been demonstrated to be capable of orchestrating EMT programs during embryonic development and in cancer. Emerging data suggest extensive crosstalk among these transcription factors, allowing them to form a signaling network that is responsible for establishing and maintaining mesenchymal cell phenotypes (Peinado et al. 2007, Moreno-Bueno et al. 2008a). Furthermore, some of these transcription factors, including Twist1, play a part in overcoming cellular senescence (Ansieau et al. 2008) and in generating tumorigenic cancer stem cells (Mani et al. 2008). This notion is reinforced by the actions of the receptor c-kit, an important agent for maintaining the stem cell state in the haematopoietic system, which induces Slug expression, as demonstrated by genetic data in mice (Perez-Losada et al. 2002) and in humans (Sanchez-Martin et al. 2002). In the same, microRNAs (miRNAs) have been recognized as essential regulators of stem-cell function, differentiation and embryonic development (Stefani and Slack 2008, Grosshans and Filipowicz 2008).

Highlighting the importance of EMT in therapeutic resistance, cancer cells with stem cell characteristics have been found to be enriched in the residual tumors remaining after standard chemotherapeutic treatments (Yu et al. 2007, Li et al. 2008). EMT and acquisition of cancer stem cell features have also been associated with increased resistance to apoptosis in breast cancer and mammary cells (Robson et al. 2006, Yu et al. 2007) as we also have reported in liver cells (Valdes et al. 2002, del Castillo et al. 2006, Caja et al. 2009).

Studies of neoplastic tissues have provided evidence of self-renewing, stem-like cells within tumors, which have been called cancer stem cells (CSCs). CSCs constitute a small minority of neoplastic cells within a tumor and are defined operationally by their ability to seed new tumors. For this reason, they have also been termed “tumor-initiating cells” (Reya et al. 2001). During the process of tumor metastasis, which is often enabled by EMTs (Thiery 2003), disseminated cancer cells would seem to require self-renewal capability, similar to that exhibited by stem cells, in order to spawn macroscopic metastases. This raises the possibility that the EMT process, which enables cancer cell dissemination, may also impart a self-renewal capability to disseminating cancer cells. Indeed, the metastatic process is at least superficially similar to the processes that occur during tissue repair and regeneration and enable adult stem cells to exit tissue reservoirs, such as the bone marrow, enter and survive in the circulation, and exit into secondary tissue sites, where they proliferate, differentiate and participate in tissue reconstruction (Kondo et al. 2003). 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, Yamashita et al. 2008b, Caja et al. 2011a). Cancer stem cells are more likely to metastasize and are more frequently detected in the circulation and in micrometastases. However, macroscopic distant metastases are more frequently composed of more differentiated epithelial cancer cells. This can be explained by the reversal of EMT through MET after micrometastases grow, due to local selective pressure for the outgrowth of cancer cells with more epithelial features or to the absence of EMT-inducing signals at sites of dissemination (Polyak and Weinberg 2009).

3. Snail

3.1. Snail family and development

The Snail gene in Drosophila (sna), first identified during analysis of dorso-ventral patterning, is a zinc finger gene with repressor function required for mesoderm formation (Boulay et al. 1987, Leptin 1991). Snail protein (Snai1) is part of a superfamily of transcription factors composed of the Snai and the Scratch family (Nieto 2002, Barrallo-Gimeno and Nieto 2009). The Snai family contains two more members: Slug (Snai2 (Cohen et al. 1998)) and Smuc (Snai3 (Katoh 2003)) on chromosomes 8 and 16, respectively. Isolation of other Snail homologues in different species including the human indicated a high degree of conservation in coding sequence and predicted protein pointing towards a conserved role in early morphogenesis (Manzanares et al. 2001, Knight and Shimeld 2001). It seemed that the Scratch genes constituted a closely related family that grouped together and was separate from the other C_2H_2 zinc-finger proteins (Manzanares et al. 2001, Knight and Shimeld 2001). Indeed, three Snail (snail, escargot and worniu) and three Scratch (scratch, scratch-like1 and scratch-like2) genes were found in Drosophila, whereas two representatives of each group were evident in vertebrates. Recently it has confirmed that Snail and Scratch proteins are a separate subgroup of C_2H_2 zinc-finger containing proteins, supporting a common origin for Snail and Scratch genes in evolution (Barrallo-Gimeno and Nieto 2009).

To exert its function as repressor, Snail nuclear import is mediated by importins which recognize a nuclear localization signal that consists of basic residues situated in the zinc finger region (Mingot et al. 2009). Inside the nucleus Snail is required to form ternary complexes with co-repressors via the Snag domain. Different ternary complexes have been described which consist of Ajuba (a novel cytosolic LIM protein) as mediator for the interaction of Snail with PRC2 (polycomb repressor complex 2) (Herranz et al. 2008)), 14-3-3 and PRMT5 (protein arginine methyltransferase 5) (Hou et al. 2010)), Sin3A for the interaction with HDAC1/HDAC2 (Histone deacetylases 1 and 2 (Peinado et al. 2004), and LSD1 (Lysine-specific demethylase 1) for the interaction with the co-

repressor complex CoREST (Lin et al. 2010). Binding to DNA occurs via E-box elements (5'-CACCTG-3') found in the promoter region of different genes including the E-cadherin gene CDH1 (Batlle et al. 2000, Cano et al. 2000).

The regulation of Snail activity mainly involves the central part of the protein which contains most sites for post-translational modification: serine phosphorylation sites (Ser92, 96, 100, 104, 107) in the SRD (signal-responsive domain) as well as two lysine oxidation sites (Lys98 and 137), and the NES (nuclear export signal) for Crm1-dependent nuclear export. Two additional serine phosphorylation sites are found N-terminal at Ser11 and C-terminal at Ser246. Phosphorylation of Ser 96, 100, 104 and 107 by GSK3β (glycogen synthase kinase 3 beta) is associated with nuclear export, ubiquitination by β-TrCP1 or FBXL14 (E3 ubiquitin ligases) and proteasomal degradation (Domínguez et al. 2003, Zhou et al. 2004a, Vinas-Castells et al. 2010). Snail up-regulation in cells has been reported as a result of diverse stimuli including cytokines (interleukin-6), growth factors (TGF-β, FGF, PDGF, EGF) and activation of their corresponding receptor tyrosine kinases as well as activation of developmental signaling pathways such as Wnt and Hedgehog. Notably, TGF-β has been described as important EMT trigger leading to HMGA2 (high mobility group A2 gene) and Smad binding at the Snai1 promoter (Thuault et al. 2008).

Snail protein functions as E-cadherin repressor and is essential during early developmental stages (Cano et al. 2000, LaBonne and Bronner-Fraser 2000). Snail genes are fundamental for metazoan embryonic development in processes involving large-scale cell movements, such as gastrulation in species from diploblasts to humans or neural crest formation in vertebrates (Barrallo-Gimeno and Nieto 2005, Peinado et al. 2007). Snail1 also acts on developing and adult bones, regulating their length or controlling mineralization, its deregulation also leads to the development of achondroplasia or osteomalacia, respectively (de Frutos et al. 2007, de Frutos et al. 2009). The Scratch genes are expressed in the nervous system of all species analyzed to date, although their specific functions have still to be defined (Roark et al. 1995, Nakakura et al. 2001, Marin and Nieto 2006).

3.2. Snail and tumor progression

Snail is re-expressed during adult life in tissue repair and their aberrant activation in the adult leads to several known pathologies, including fibrosis (Boutet et al. 2006), tumor progression and recurrence (Moody et al. 2005a, Peinado et al. 2007). Indeed, forced Snail1 expression in normal kidneys is sufficient to generate EMT and fibrosis, leading to organ failure in transgenic mice (Boutet et al., 2006; López-Novoa and Nieto, 2009). Snail factors regulate cell–cell adhesion and trigger the epithelial-to mesenchymal transition (EMT) by repressing the gene expression of E-cadherin (Horiguchi et al. 2009), converting almost static epithelial cells into motile and invasive mesenchymal

cells with stem cell properties (Barrallo-Gimeno and Nieto 2005, Peinado et al. 2007, Mani et al. 2008).

Some of the challenges in this area may be due to the transient and dynamic nature of tumor-associated EMT. Also, it has been proposed that Snail is required as initial trigger in EMT, whereas maintenance of the phenotype is taken over by other factors potentially leaving behind a mesenchymal cell devoid of Snail expression (Peinado et al. 2007). Another intriguing recent observation is the presence of Snail in tumor-associated stroma as it might be an informative indicator of tumor (Franci et al. 2009), however the lack of good monoclonal antibodies against this protein has precluded a definitive analysis of Snail1 protein.

Expression of Snai1 at the transcript level has been detected in benign conditions such as tissue fibrosis (Sato et al. 2003, Jayachandran et al. 2009), a range of malignant neoplasms (Cheng et al. 2001, Takeno et al. 2004) and in normal tissue adjacent to tumor (Pena et al. 2009). Early studies based on detection of the Sna1 transcript found associations with lymph node metastasis (Cheng et al. 2001) and malignant effusion (Elloul et al. 2005) in breast cancer. A mouse model reported by Moody and collaborators implicated Snail expression with mammary cancer recurrence (Moody et al. 2005b). Other studies described associations between elevated Snai1 transcript levels and hypoxia in ovarian cancer (Imai et al. 2003), downregulation of Vitamin D Receptor in colon cancer (Pena et al. 2005), invasion and distant metastasis in esophageal squamous cell carcinoma (Takeno et al. 2004), invasiveness (Sugimachi et al. 2003) and poor prognosis in hepatocellular carcinoma (Miyoshi et al. 2005) and spindle cell phenotype in synovial sarcoma (Saito et al. 2004). However, it has been pointed out that transcript levels may not correlate well with Snail protein which is tightly regulated and subject to a short half-life previously reported as approximately 25 minutes (Zhou et al. 2004a). Also, transcript levels may be confounded by Snail expression in the stromal tumor component if no micro-dissection is performed (Peinado et al. 2007). Immunohistochemical detection of Snail has been documented for a range of cancers including the upper gastrointestinal tract (Usami et al. 2008, Kim et al. 2009), head and neck (Peinado et al. 2008, Yang et al. 2008b, Schwock et al. 2010), colorectum (Franci et al. 2009) and sarcomas (Franci et al. 2006) among others.

Consequences of Snail up-regulation not only include the repression of E-cadherin transcription, but the negative as well as positive control of a series of genes involved in a range of biological functions such as cell-cell adhesion, cell-extracellular matrix interaction, cell polarity, cytoskeleton, cell cycle, survival, and angiogenesis leading to a phenotypic shift towards more mesenchymal cellular characteristics (De Craene et al. 2005, Higashikawa et al. 2008). In the context of tumor-associated EMT these mesenchymal-like characteristics have been correlated with a greater resistance to different therapeutic modalities (Kajita et al. 2004, Kurrey et al. 2009), escape from attack by the immune system (Kudo-Saito et al. 2009) and adoption of a cancer stem cell phenotype (Mani et al. 2008, Morel et al. 2008). Our group previously published

that in MDCK cells Snail attenuates the cell cycle and confers resistance to cell death induced by the withdrawal of survival factors and by pro-apoptotic signals (Vega et al. 2004). However, further work is needed to better understand the Snail's effects on apoptosis.

4. SPARC

4.1. Secreted protein acidic and rich in cysteine (SPARC) family and physiological functions

This growing family of secreted extracellular matrix-associated proteins comprises thrombospondins, tenascins, osteopontin, CNN proteins and SPARC. Although they have no structural homology, they all share the functional property of modulating cell–matrix interactions. Secreted protein acidic and rich in cysteine (SPARC, also known as osteonectin or BM-40) is the prototype of this group of microenvironmental proteins.

Structure–function studies using synthetic SPARC peptides previously identified four individual SPARC sequences that regulate cell proliferation: domain peptides 2.1 (follistatin-like domain, 55–74aa in green in Figure i.4) and 2.3 (K)GHK angiogenic peptide, 114–130aa in black in Figure i.4) that inhibit and stimulate DNA synthesis, respectively; and EC domain peptides 4.0 (248–285 aa) and 4.2 (255–274aa in yellow in Figure i.4) that both inhibit DNA synthesis (Funk and Sage 1991, Funk and Sage 1993, Sage et al. 1995, Kupprion et al. 1998, Motamed et al. 2002).

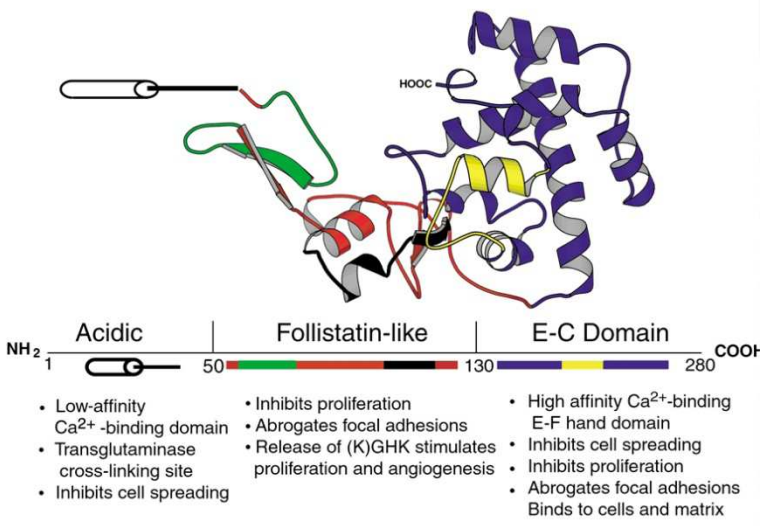


Figure i.4. Structure of SPARC protein from Bradshaw and Sage 2001.

SPARC N-terminal domain binds hydroxyapatite and calcium, a follistatin-like domain that stabilizes two weakly interacting modules, and a C-terminal domain that binds calcium and contains structural similarity to the Kazal family of serine proteases (Tai

and Tang 2008). Kosman and collaborators demonstrate that the motif of SPARC that inhibits DNA synthesis is not a nuclear localization signal (NLS) but the manipulation of the putative NLS generates a SPARC that is a more potent inhibitor of DNA than the wild-type SPARC that might be a useful therapeutic tool to quiesce proliferative tissues (Kosman et al. 2007). Additionally, SPARC peptides generated by protease cleavage can modulate cell shape, control cell proliferation, and stimulate cell migration (Tai and Tang 2008). Collectively, these studies have shown SPARC to be a structurally complex protein, one that contains multiple independent protein sequences possessing redundant and/or antagonist activities.

The first word in the acronym SPARC establishes that this protein is secreted and is therefore extracellular. Primarily, SPARC is classified as a matricellular protein that is spatially and temporally regulated during development and expressed at high levels in remodeling tissues (Bradshaw and Sage 2001b, Brekken and Sage 2001, Tartare-Deckert et al. 2001). SPARC interacts with several extracellular matrix components and functions as a de-adhesive molecule (Murphy-Ullrich 2001), as inhibitor of cell spreading (Delostrinos et al. 2006), as a cell cycle inhibitor (Bradshaw and Sage 2001b, Fenouille et al. 2010), and as a modulator of cytokine and growth factor activities. For instance, SPARC binds platelet-derived growth factor (PDGF) (Raines et al. 1992) and vascular endothelial growth factor (VEGF) (Kupprion et al. 1998), preventing their signaling through their respective receptors. In these examples, the mechanism of action of SPARC is predicated upon binding to an extracellular signaling mediator, transducing or blocking an intracellular signaling cascade. SPARC is known to attach to the extracellular matrix (ECM) through interactions with collagen-I and other collagens found in the ECM with high affinity (Termine et al. 1981, Mayer et al. 1991, Maurer et al. 1995, Yan and Sage 1999).

SPARC mediates diverse cellular processes such as de-adhesion, migration and inhibition of proliferation via its regulation of cell–matrix interactions and extracellular matrix production (Brekken and Sage 2001). SPARC is involved in normal tissue remodeling processes such as bone resorption, endothelial cell migration, angiogenesis, chaperone activity, wound healing and tissue injuries, as well as inflammation and cancer progression (Bradshaw and Sage 2001b, Sangaletti and Colombo 2008, Clark and Sage 2008). Overproduction of collagens in systemic sclerosis is associated to SPARC as its specific inhibition in normal human fibroblasts attenuated the profibrotic effect of TGF-β, however it's not associated to TβRI nor Smad3 expression (Zhou et al. 2006).

SPARC is able to remain outside of cells to complete its primary tasks. The exception to these findings is the discovery that SPARC promotes formation of fibronectin-induced stress fibers via an intracellular interaction with integrin-linked kinase (ILK) (Barker et al. 2005). Only one receptor has been identified as binding and internalizing SPARC, stabilin-1, but this pathway is functional only in activated macrophages, and its purpose is to immediately target SPARC for degradation in lysosomes (Kzhyshkowska et al. 2006). Yet, SPARC has reportedly been found in cell nuclei. Both immunoblotting and

immunofluorescent staining of cultured embryonic chicken cells and bovine aortic endothelial (BAE) cells detected SPARC in nuclei (Gooden et al. 1999). SPARC persisted in the nuclei even after nuclear matrix unmasking the cells, demonstrating a strong interaction between SPARC and the nuclear matrix (Gooden et al. 1999). The nuclear localization of SPARC has not been found to be constitutive, but rather has been variously identified as being cell-cycle-dependent (Gooden et al. 1999) or concentration-dependent (Yan et al. 2005). No study has yet identified any significant bioactivity in association with the nuclear localization of SPARC.

4.2. SPARC and EMT

Importantly, SPARC expression in melanoma cells has been associated with the acquisition of mesenchymal characteristics. SPARC is an important regulator of the E-cadherin to N-cadherin switch, increases Vimentin expression and induces TGF-β secretion (Bassuk et al. 2000, Robert et al. 2006, Girotti et al. 2011). Melanoma cells that overexpress SPARC have reduced E-cadherin expression (Smit et al. 2007) and SPARC knockdown in melanoma cells downregulates N-cadherin levels (Sosa et al. 2007). On the one hand SPARC induces up-regulation of Snail during melanoma development and depletion of SPARC decreases Snail expression (Robert et al. 2006). On the other hand it has been published that SPARC expression is regulated by Snail, Slug and E47 factors in MDCK cells (Moreno-Bueno et al. 2006) and silencing of Snai1 in breast carcinoma cells induces a decrease in SPARC levels concomitant with reduced in vitro and in vivo invasive behavior (Olmeda et al. 2007). E- to N-cadherin switching, dissociation of β-catenin from the membrane, and increased expression of Snail and SPARC in the sarcomatous component of Sarcomatoid renal cell carcinomas indicate that it is an example of EMT (Conant et al. 2011). In addition, SPARC regulates the expression of other genes directly involved in EMT, such as Vimentin and member C/interleukin-like EMT-inducer. Moreover, SPARC-induced N-cadherin expression gives melanoma cells the ability to transmigrate through endothelial cells (Girotti et al. 2011). As reported by Roberts and collaborators, the COOH-terminal extracellular Ca^{2+} binding module (EC) of SPARC is dispensable mediating E-cadherin loss and that the NH_2-terminal acidic and follistatin-like modules contain determinants that are responsible for inhibiting E-cadherin expression in melanocyres (Robert et al. 2006). The EC mutant fails to inhibit cell spreading and attachment, suggesting that SPARC induces E-cadherin repression independently of its well-established counteradhesive property as also has been published the role of the EC domain in the anti-spreading activity of SPARC in urothelial cells (Delostrinos et al. 2006). It's been demonstrated that SPARC-EC inhibition of cell proliferation needs in part TGF-β receptors and Smad2/3 nuclear translocation in Mv1Lu epithelial cells (Schiemann et al. 2003). SPARC antiadhesive ability might result from directly interfering with the binding of cell surface integrins to components of the ECM (Clark and Sage 2008). Moreover, its common appearance during metastasis has led some to believe that it may fulfill several aspects of Pagets

"seed and soil" hypothesis (Paget 1989, Fidler and Poste 2008), whereby it facilitates tumor cell, stromal cell, and ECM interactions (Fidler 2003, Framson and Sage 2004).

Although SPARC induces numerous events associated with mesenchymal transition, expression of SPARC is not sufficient to enhance N-cadherin or other markers associated with melanoma progression, such as h3 integrin, MMP-2, MelCAM, or CD44v6 (Robert et al. 2006), indicating the possible involvement of additional genetic or epigenetic events in this process. SPARCs involvement in EMT has been reported in the context of tumor progression, where microarray profiling of melanoma and breast cancers revealed its up-regulation in tumor samples displaying an EMT phenotype (Alonso et al. 2007, Lien et al. 2007).

4.3. SPARC and tumor progression

The role of matrix stiffness in tumor progression has been recently highlighted (Assoian and Klein 2008, Levental et al. 2009). Members of the matricellular protein family are known to influence interactions between malignant cells and their microenvironment, and act as potent modulators of cellular functions (Bornstein and Sage 2002, Clark and Sage 2008).

The role of SPARC in cancer biology is complex and dependent upon the origin of the tumor cells and the environment in which tumors evolve. SPARC exerts tumor suppressive activities in neuroblastomas, pancreatic, colorectal and ovarian cancers, and certain myelodysplastic syndromes and acute myeloid leukemia (Clark and Sage 2008, Podhajcer et al. 2008).

Interestingly, tumor types associated with mesenchymal transformation, such as the highly aggressive basal-like and metaplastic breast carcinomas, express high levels of SPARC, which is a marker of poor outcome (Lien et al. 2007). This relationship of SPARC with bad prognosis correlates with SPARC behavior in vitro and in vivo assays. For example, in glioma cells, SPARC induction has been shown to promote cell motility and invasion along with an increase in certain matrix metalloproteinases in the extracellular milieu (Golembieski et al. 1999, Schultz et al. 2002). The progression to malignant melanoma coincides with the altered expression of cell–matrix and cell–cell communication molecules such as SPARC (Haass et al. 2005, Miller and Mihm 2006). SPARC has been shown to be an important marker for melanoma invasion and metastasis, and in patients with melanoma its expression correlates with the aggressiveness of the tumor and poor survival (Massi et al. 1999, Sturm et al. 2002, Alonso et al. 2007). Autocrine SPARC signaling mediates melanoma invasion through repression of E-cadherin and induction of mesenchymal transition (Robert et al. 2006). In addition, reducing SPARC levels in human melanoma cells was shown to delay tumor growth in mouse xenografts and SPARC produced by melanoma cells regulates their in vitro proliferative capacity (Ledda et al. 1997, Prada et al. 2007). SPARC also promotes aggressive tumor phenotype and metastatic behavior in prostate cancer (Clark and

Sage 2008, Podhajcer et al. 2008). The role of SPARC in hepatocellular carcinoma is rather ambiguous by acting either as a tumor promoter (Atorrasagasti et al. 2010) or tumor suppressor as found in patients at advanced stages of disease (Keating and Santoro 2009). Recent data indicates that the upregulation of SPARC is caused by demethylation during HCC progression (van Zijl et al. 2011) and this could explain its dual role in HCC. Likewise, SPARC may promote metastasis (Minn et al. 2005, Clark and Sage 2008), not only because it increases motility and migratory behavior, but also because SPARC upregulation may allow cancer cells to survive competitive interactions (Portela et al. 2010) once they seed into soil tissues.

The search for molecular mechanisms that explain SPARC tumor cell activity has been unsuccessful. There seems to be no consensus of the pathways affected by SPARC in normal and tumor cells. SPARC features in normal cells seem to be explained by SPARC interaction with integrin-β1 (Weaver et al. 2008, Nie et al. 2008), but were not reproduced in tumor cells. No actual ligand was described for SPARC in tumor cells, even when several reports related SPARC to focal adhesion kinase- and integrin-linked kinase-mediated signaling pathways within glioma and melanoma cells (Thomas et al. 2003, Smit et al. 2007, Shi et al. 2007a, Fenouille et al. 2010).

5. The Transforming growth factor beta (TGF-β)

5.1. Soluble factors of the TGF-β family proteins

Since the discovery of the first member of the transforming growth factor-β (TGF-β) superfamily in 1978 (de Larco and Todaro 1978), a growing number of related members have been identified and functionally characterized in vertebrates and invertebrates. So far, more than 40 members of this family are known, which have, in common, their dimeric structure and the presence of a cysteine knot structural motif (Galat 2011). These proteins cluster in several subfamilies, such as TGF-βs, BMPs (bone morphogenetic proteins), GDFs (growth and differentiation factors), MIF (Müllerian inhibitory factor), activins or inhibins.

As revised in (Santibañez et al. 2011), among the TGFβs, six distinct isoforms with a variable degree of homology have been discovered, although only the TGF-β1, TGF-β2 and TGF-β3 isoforms are expressed in mammals (Lyons and Moses 1990). BMPs were originally identified as a family of proteins that induced the formation of bone and cartilage when implanted at ectopic sites in rats. Members of the BMP family have been found in vertebrates as well as in invertebrates and are known to exhibit a wide range of biological effects on various cell types (Miyazono et al. 2010, Rider and Mulloy 2010). BMPs regulate the transcription of several genes involved in osteogenesis, neurogenesis and ventral mesoderm specification. Members of the BMP family can be classified into several subgroups, including the BMP2/BMP4 group, the BMP5–BMP8

group, the OP-1 (osteogenic protein-1) group and the BMP9/BMP10 group (Miyazono et al. 2010, Rider and Mulloy 2010). GDFs are classified within the BMPs family and include, at least, 11 components: GDF1–GDF3, GDF5–GDF11 and GDF15 (Moustakas and Heldin 2009). MIF, also known as AMH (anti-Müllerian hormone) or MIS (Müllerian inhibitory substance) has been mainly studied for its regulatory role in male sex differentiation (Nef and Parada 2000). Activins are structurally related proteins involved in the control of cell proliferation, differentiation, apoptosis, metabolism, homoeostasis, differentiation, immune response and endocrine function (Xia and Schneyer 2009). Activins are secreted as homodimers or heterodimers of inhibin β-subunits. Although four β-subunit genes (βA, βB, βC and βE) have been described in humans, only dimers composed of βA/βA (activin A), βB/βB (activin B) and βA/βB (activin AB) subunits have been shown to be biologically active (Stenvers and Findlay 2010). Inhibins were originally characterized as proteins produced by the gonads that act in an endocrine manner to negatively regulate FSH synthesis and secretion from the anterior pituitary. As such, inhibins are essential for normal reproductive and endocrine function (Stenvers and Findlay 2010). Inhibins are closely related to activins. Inhibins are disulfide-linked heterodimers comprising an α-subunit and either a βA or βB subunit to form inhibin A and inhibin B respectively (Makanji et al. 2007).

5.2. TGF-β receptors

All TGF-β family members bind cell-surface serine/threonine kinase receptors types I and II (TβRI and TβRII respectively), which form heteromeric complexes in the presence of dimerized ligands. Seven TβRIs, also named ALKs (activin-like receptor kinases), as well as five different TβRIIs have been described (see Table i.I).

Receptor type	Ligand	R-Smad
Type I receptor		
ALK1/ACVRL1	TGF-β, BMP9 and BMP10	Smad1, Smad5 and Smad8
ALK2/ACVR1	BMPs and GDFs	Smad1, Smad5 and Smad8
ALK3/BMPR1A	BMPs	Smad1, Smad5 and Smad8
ALK4/ACVR1B	Activins, myostatin/GDF8 and GDF11	Smad2 and Smad3
ALK5/TGFBR1	TGF-βs, myostatin/GDF8 and GDF11	Smad2 and Smad3
ALK6/BMPR1B	BMPs	Smad1, Smad5 and Smad8
ALK7/ACVR1C	BMP16/nodal	Smad2 and Smad3
Type II receptor		
TGFBR2/TBRII	TGF-βs	
BMPR2/BMPRII	BMPs and GDFs	
ACVR2/ActRIIA	Activins, BMP2, BMP4, BMP7 and GDFs	
ACVR2B/ActRIIB	Activins, BMP2, BMP4, BMP7, GDFs and BMP16/Nodal	
AMHR2/AMHRII	AMH/MIF/MIS	
Type III receptor		
Betaglycan	TGF-β1, TGF-β2, TGF-β3, activin-A, BMP2, BMP4, BMP7 and GDI	
Endoglin	TGF-β1, TGF-β3, activin-A, BMP2, BMP7 and BMP9	

Table i.I. TGF-β receptors, their ligands and R-Smads. From Santibañez et al. 2011.

In addition, TGF-β ligands may interact with the co-receptors endoglin and betaglycan, known as TβRIIIs (type III TGF-β receptors) (Gatza et al. 2010, Bernabeu et al. 2009, Shi and Massague 2003). Soluble ligands bind first to the constitutively active TβRII, followed by the interaction and phosphorylation of a GS (glycine/serine)-rich domain of the TβRI to produce an activated ligand–receptor complex (Kang et al. 2009). Then, the activated TβRI phosphorylates the downstream effector Smads. Both type I and II kinase receptors are themselves phosphorylated at tyrosine and serine/threonine residues, probably implicated in a crosstalk activity regulation of a variety of signal transduction pathways.

The activity of TGF-β kinase receptors can be regulated by the auxiliary TβRIII endoglin or betaglycan (Gatza et al. 2010, Bernabeu et al. 2009). Endoglin and betaglycan are type I integral membrane proteins with large extracellular domains and short cytoplasmic domains lacking kinase signaling motifs. The ubiquitous betaglycan binds with high affinity to several members of the TGF-β family, including TGF-β1, TGF-β2, TGF-β3, activin-A, BMP2, BMP4, BMP7 and GDF5 (Gatza et al. 2010). Betaglycan increases ligand binding to the respective cognate TβRIs and TβRIIs to modulate their signaling (Gatza et al. 2010, Kang et al. 2009, Lewis et al. 2000). At variance with betaglycan, endoglin is predominantly expressed in vascular endothelial cells, a cell type that has little or no betaglycan expression. Endoglin interacts with TGF-β1, activin-A, BMP2 and BMP7, requiring the presence of the corresponding signaling receptors; by contrast, endoglin can bind directly to TGF-β3 and BMP9 independently of the kinase receptors (Bernabeu et al. 2009). Endoglin enhances TGF-β1-, BMP7- and BMP9-dependent Smad1/Smad5 responses, while it inhibits the TGF-β/Smad3 pathway (Gatza et al. 2010, Bernabeu et al. 2009, Santibanez et al. 2007, Blanco et al. 2005).

5.3. TGF-β canonical Smad signaling pathway

The Smad family has eight members, two TGF-β R-Smads (Smad2 and 3), three bone morphogenetic protein (BMP) R-Smads (Smad1, 5 and 8), one Co-Smad (Smad4) and two I-Smads (Smad6 and 7). The R-Smads and the Co-Smad have conserved Mad-homology 1 (MH1) and MH2 domains connected by a linker, whereas the I-Smads have an MH2 domain but no distinct MH1 domain. The Smad family members are well conserved and almost all vertebrates have all eight members (Huminiecki et al. 2009).

The R-Smads and the Co-Smad bind DNA via a β-hairpin structure in their MH1 domains, except for Smad2, which has an inserted sequence in this region preventing DNA binding (Shi et al. 1998). Smad3 and 4 bind with selectivity to a CAGA-motif but with low affinity. The MH1 domain furthermore mediates interactions with other transcription factors, including activating transcription factor 2 (ATF2), Sp1, Jun and transcription factor E3 (TFE3), which helps to stabilize a DNA-binding complex (Moustakas and Heldin 2009, Shi and Massague 2003). The MH2 domain of R-Smads is

responsible for the interactions with the type I receptors. The linker regions between the MH1 and MH2 domains also have important functions in Smad regulation, since they contain PY motifs that bind ubiquitin ligases and sites of phosphorylation targeted by various kinases (Heldin and Moustakas 2011).

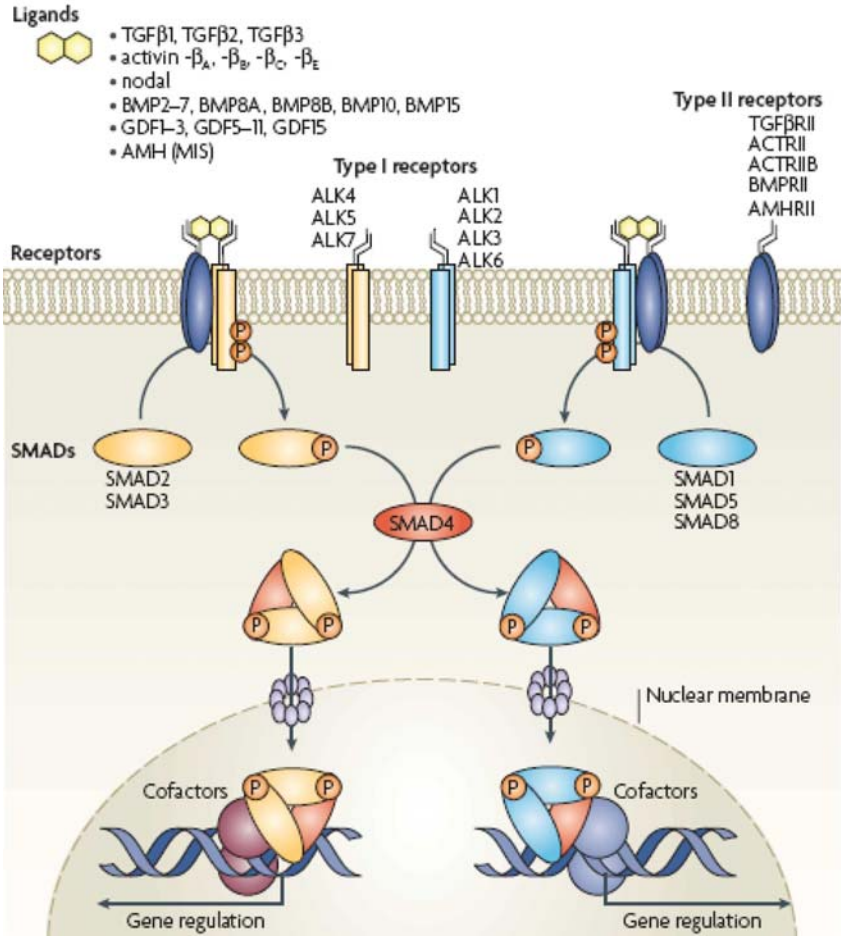


Figure i.3. TGF-β Canonical Smad signaling. From Schmierer and Hill 2007.

Transforming growth factor-beta (TGF-β) superfamily members signal via membrane-bound dual specificity kinase receptor complexes, acting both as tyrosine and serine/threonine kinase receptors. Upon ligand binding, a heterotetrameric receptor complex of two type I (TβRI) and two type II receptors (TβRII) is formed; the constitutively active TβRII phosphorylates and activates TβRI (Heldin et al. 2009, Kang et al. 2009, Massague 2008). The activated serine/threonine kinase TβRI phosphorylates Smad2 and Smad3, which form hetero-oligomers with Smad4. They translocate from the cytoplasm to the nucleus, where they regulate transcription of target genes (Schmierer and Hill 2007, Bierie and Moses 2006, Zavadil and Bottinger 2005) .

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, thus allowing the Smads to become available for export to the cytoplasm (Pardali and Moustakas 2007).

Some receptor-associated proteins were identified by their ability to facilitate the Smad interactions with the receptor complexes and, consequently, their activation. Examples of these adaptors are SARA (Smad anchor for receptor Activation), a scaffold protein that, in mammalian cells, stabilizes the binding of Smad2 and Smad3 to TβRI and localizes the receptors in EEA1-positive early endosomes. Endofin, a structural homolog to SARA, can interact with Smad1, a mediator of BMP signaling and might have a similar role as SARA in BMP receptor signaling (Shi et al. 2007b) and binds to TβRI and Smad4 facilitating TGF-β signaling (Chen et al. 2007). Other adaptors of Smad interaction with type I receptors are Axin, Dab2 and Dok-1. Axin, an inhibitor and scaffold protein in Wnt-induced β-catenin signaling, 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).

For an efficient TGF-β signaling through Smads, TGF-β ligands induce receptor internalization in endosomes and association with caveolin, a protein present in plasma-membrabe invagionations. The caveolin-positive lipid-raft compartment is required for receptor turnover and regulates receptor availability and R-Smad activation (Derynck and Zhang 2003).

Smads can be regulated by different mechanisms as revised by Helding and collaborators. A number of different kinases can phosphorylate Smads acting as agonists or antagonists of their signaling. Their stability can be regulated by ubiquitin-mediated degradation in proteasomes and lysosomes and by sumoylation can be protected from ubiquitination. There's a negative feedback control of Smad signaling through Smad7 by competing with R-Smads for receptor binding thus inhibiting their phosphorylation, by recruiting ubiquitin ligases to the receptors thus promoting their ubiquitination and proteasomal degradation, by recruiting phosphatases to the receptors thus promoting their dephosphorylation and deactivation and by interfering with the binding of Smad complexes (Heldin and Moustakas 2011).

5.4. TGF-β non-Smad pathways

In addition to the well-characterize Smad signaling pathways, TGFβ also activates non-Smad signaling pathways such as TAK1 (TGFβ-associated kinase 1), Erk (extracellular signal regulated kinase), p38, MAPK (mitogen-activated protein kinase), and Akt. These pathways have been implicated in the regulation of apoptosis, cell migration and EMT

among others as it's been reported by our group and others (Martínez-Palacián et al. 2012, Sancho and Fabregat 2010, Franco et al. 2010, Ikushima and Miyazono 2010, Fabregat 2009, Zhang 2009, Heldin et al. 2009, Massague 2008, Fabregat et al. 2007, Murillo et al. 2007, Caja et al. 2007, Murillo et al. 2005, Valdes et al. 2004, Valdes et al. 2002, Wakefield and Roberts 2002, Lo et al. 2001, Fabregat et al. 2000, Bakin et al. 2000).

The Smad receptors can act as platforms for the initiation of the Erk-MAPK signaling pathway because of their dual specificity acting as both tyrosine and serine/threonine kinases.

The autophosphorylation of TβRII at tyrosine residues results in the recruitment of Src homology 2 (SH2)-domain proteins to TβRII in analogy with the signaling events evoked by the ligand-induced activation of tyrosine kinase receptors (Schlessinger 2000). Src-mediated phosphorylation of TβRII Y284 results in the recruitment of the SH2 domains of growth factor receptor binding protein 2 (Grb2) and Src homology domain 2-containing protein (Shc) to TβRII, thereby associating these adapter proteins with p38 MAPK activation (Galliher and Schiemann 2007).

Activated TβRI uses its intrinsic tyrosine kinase activity to phosphorylate Shc directly on its tyrosine and serine residues. Phosphorylated Shc associates with TβRI via its phosphotyrosine-binding domain and, in turn, recruits Grb2 and SOS, activating Erk-MAPK (Lee et al. 2007). Another way for Erk to negatively influence the canonical TGFβ-induced activation of the Smad pathway and EMT induction has recently been demonstrated. The Erk-MAPK pathway decreases TGFβ-induced activation of Smad3 because of a specific decrease in the cell-surface levels of TβRI (Liu et al. 2009).

TβRI has been found to harbour a consensus binding site for the ubiquitin ligase tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6); upon TGFβ binding TRAF6 activates the TGFβ activated kinase 1 (TAK1)—p38 mitogen activated protein (MAP) kinase pathway (Sorrentino et al. 2008, Yamashita et al. 2008a). It's been recently reported that TRAF6 ubiquitinates TβRI to promote its cleavage and nuclear translocation which has an important impact in cancer development (Mu et al. 2011). Interestingly, TNF-alpha converting enzyme (TACE) has been descrived to cleave TβRI in its extracellular domain, which was demonstrated to cause a loss of TGFβ-induced inhibition of proliferation of cells (Liu et al. 2009). Our group has demonstrate that TACE activity is required for EGFR ligand proteolysis and activation (Murillo et al. 2005, Caja et al. 2007) and the inability of adult hepatocytes to induce survival signals appeared to be due to the very low expression of both Akt and TACE observed (Caja et al. 2007).

TGF-β is known to cause the activation of the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway (Bakin et al. 2000), although the underlying molecular mechanisms are not well characterized. Both TβRII and TβRI appear to be required for the activation of the PI3K pathway, and TβRI has also been found to associate with the p85 subunit of

PI3K (Yi et al. 2005). The recent identification of TGFβ-induced regulation of the mTOR pathway suggests that TGFβ utilizes this pathway to regulate cell survival, metabolism, migration, and invasion (Lamouille and Derynck 2007, Lamouille and Derynck 2011).

We have described that TGF-β induced apoptosis is counteracted by pre-incubation of fetal rat hepatocytes with EGF in rat fetal hepatocytes (Fabregat et al. 1996) which, through the activation of the PI3K pathway, blocked cytochrome c release (Fabregat et al. 2000). Afterwards we demonstrate that TGF-β induces antiapoptotic signals through production of EGF Receptor (EGFR) ligands that stimulate an autocrine loop of EGFR activation (Caja et al. 2007) and EGFR downstream survival effectors: c-Src, PI3K and Akt (Valdes et al. 2004, Murillo et al. 2005). One of the main characteristic facts reported during the development of liver tumors is the acquisition of resistance to apoptosis and activation of survival signals, favoring an uncontrolled growth of tumoral cells (Fabregat et al. 2007). Thus, the over-activation of the EGFR might contribute to the tumorigenic capacity of FaO rat hepatoma cells and, in fact, the incubation with EGFR inhibitors sensitizes them to TGF-β-induced cell death (Caja et al. 2007). It is interesting to point out that some reports suggest the effectiveness of EGFR inhibitors as potential therapy in hepatocellular carcinoma (HCC) models both in vivo and in vitro (Hopfner et al. 2004, Schiffer et al. 2005, Liu et al. 2007b). It has been described that EGF impairs TGF-β-induced apoptosis in hepatocytes and hepatoma cells (Fabregat et al. 2000, Shima et al. 1999a), and that the EGFR transactivation by TGF-β mediated pro-survival effects of this cytokine in different cell lines, including FaO rat hepatoma cells (Caja et al. 2007, Docherty et al. 2006, Murillo et al. 2005, Vinals and Pouyssegur 2001).

It's been documented the potent effects that TGFβ exerts on the rapid regulation of the cytoskeleton in cells, and these effects appear to be dependent upon non-Smad signaling (Moustakas and Heldin 2005, Edlund et al. 2002). Interestingly, TGF-β causes the activation of the small GTPase Cdc42 in prostate cancer, resulting in membrane ruffling (Edlund et al. 2002). The way that TGF-β causes the activation of Cdc42 is not well understood, although the expression level of Smad7 has been suggested to be important for the activation of the TGF-β-Cdc42 pathway in prostate cancer cells (Edlund et al. 2004). Interestingly, TGF-β can specifically activate p21-activated kinase 2 (PAK2), a serine/threonine kinase that functions upstream of Cdc42 and Rac1 in fibroblasts but not in epithelial cells (Wilkes et al. 2003); TGF-β-induced activation of PI3K is required for the activation of the PAK2 pathway (Wilkes et al. 2005).

Recognition of the dual functionality of TβRI as both a serine/threonine kinase and a tyrosine kinase is an important step forward in our understanding of the versatility of the signals evoked by the activated TβR complex, particularly in tumor progression. The TGF-β-induced activation/inactivation of the small GTPases probably contributes to the regulation of cell adhesion and the TGF-β-induced migratory responses of cells; these regulatory steps might therefore be critical events leading to the EMT. Since the PI3K-Akt and Erk pathways play a crucial role in tumor progression, identification of the

regulatory mechanisms governing these pathways may provide novel drug targets for cancer treatment.

To sum up, signals induced by TGF-β are tightly regulated and specified by post-translational modifications of the signaling components, since they dictate the subcellular localization, activity, and duration of the signal.

5.5. TGF-β and growth inhibition

TGF-β is an important regulatory suppressor factor in hepatocytes, where it induces cell cycle arrest at low doses (Sanchez et al. 1995), early inhibits proliferation (Carr et al. 1986) and induces cell death (Sanchez et al. 1996, Oberhammer et al. 1992). It's well documented its ability to arrest the cell cycle at the early G1 phase and thus inhibit growth of various cell types (Massague 2004).

The regulation of growth arrest by Smads is of central importance for cell cycle arrest by TGF-β. Nuclear Smad complexes regulate target genes in an immediate-early but also a sustained manner, leading to the suppression of mitogenic transcriptional signals (c-myc and Id genes) and the induction of cell cycle inhibitory signals: p15, p21, p57 genes (Heldin et al. 2009, Massague 2004). Smad2 is a cell-autonomous negative regulator of hepatocyte growth in vitro and in vivo (Ju et al. 2006) and Smad3 has a role as a mediator of epithelial growth inhibition, although this is not universally applicable and appears to be highly context dependent (as found in mammary gland epithelial cells or transformed keratinocytes) (Ju et al. 2006). Smad3 is required for the induction of EMT in vitro as explained in the section TGF-β and cancer.

The Smad-activated transcriptional responses appear to be crucial for TGF-β-induced growth inhibition, whereas the nuclear protein TIF1γ (transcriptional intermediary factor 1γ), a monoubiquitination factor (Dupont et al. 2009), recruits activated Smad2/Smad3 to control the differentiation status of cells (He et al. 2006).

5.6. TGF-β and apoptosis

In addition to the regulation of the cell cycle, TGF-β also limits cancer formation through the activation of the apoptotic pathway.

Downstream targets for pro-apoptotic functions of TGF-β include death associated protein kinase (DAPK), growth arrest and DNA damage inducible 45β (GADD45β) and Bim (Jang et al. 2002, Takekawa et al. 2002, Ohgushi et al. 2005). For example, Bim deficiency was shown to induce follicular lymphoma and accelerate Myc‑induced generation of lymphoma in a mouse model (Egle et al. 2004). By contrast, TGF-β also exhibits anti‑apoptotic effects through the induction of the transcripcion factor Dec1 (differentiated embryo–chondrocyte expressed gene 1) under certain conditions (Ehata et al. 2007). A correlation between the expression of DeC1 and tumour grade in breast

cancer has been reported (Chakrabarti et al. 2004). TGF-β-induced DEC1 expression prevents the apoptosis of mouse mammary carcinoma cells, and a dominant negative mutant of DeC1 prevents lung and liver metastasis of breast cancer cells *in vivo* (Ehata et al. 2007). Recently, van der Heide and collaborators have identified mitogen- and stress-activated kinase 1 (MSK1) as an antagonist of TGF-β-induced cell death and report that TGF-β induction of MSK1 activity depends on both Smad4 and p38 MAPK activation (van der Heide et al. 2011).

However, in many other TGF-β-induced responses, the PI3K/Akt pathway antagonizes Smad mediated effects. For example, activation of PI3K or Akt protects cells from TGF-β-induced apoptosis and growth inhibition {Song, 2006 #3329;Valdes, 2004 #733;Shin, 2001 #5995;Chen, 1998 #2648}. This protection has been suggested to result from a physical interaction between Akt and Smad3 (Conery et al. 2004, Remy et al. 2004). Akt, which relays signals downstream of PI3K, can directly associate with Smad3. The interaction between Smad3 and Akt 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). Besides Akt, the forkhead transcription factor, FoxO, also plays a role in the antagonizing effect of PI3K/Akt on Smad-mediated transcription. TGF-β is a well-known potent growth inhibitor. Key to this function of TGF-β is its ability to suppress proto-oncogene c-Myc transcription, and to induce transcription of p15Ink4b and/or p21Cip1, which are inhibitors of G1 phase cyclin-dependent kinases (Massague 2008). In the case of induction of p15Ink4b and p21Cip1 expression, a trans-activation complex containing Smad3, Smad4, and FoxO family of transcription factors is required (Seoane et al. 2004, Gomis et al. 2006). Since FoxO proteins are targets of the PI3K/Akt pathway, Akt can inhibit nuclear localization of FoxO proteins by phosphorylating them, and thus barring them from their target genes.

Besides Smad-dependent pathways, the TRAF6-TAK1-JNK/p38 pathway is essential for TGF-β-induced apoptosis. Overexpression of TAK1 caused cells or Xenopus embryo to undergo apoptosis, whereas cells expressing the kinase-inactive TAK1 were protected from TGF-β- or BMP-induced apoptosis (Shibuya et al. 1998, Kimura et al. 2000, Edlund et al. 2003). Moreover, knockdown of TRAF6 using siRNA or treating cells with a chemical inhibitor of p38 efficiently blocked TGF-β-mediated apoptosis (Yu et al. 2002, Sorrentino et al. 2008, Yamashita et al. 2008a, Liao et al. 2001). These results indicate that the TRAF6-TAK1-JNK/p38 pathway functions in a cooperative manner with the Smad pathway in TGF-β/BMP-induced apoptosis. Most of the proapoptotic effects of TGF-β are evoked by specific transcription factors such as p53 (Zhang et al. 2006), which is co-regulated by p38 and Smad proteins.

TGF-β promotes apoptosis in hepatocytes and B-lymphocytes via Smad3-dependent transcription of the phosphatase MKP2, which enhances the pro-apoptotic effect of the Bcl-2 family member Bim (Ramesh et al. 2008) as it also does in both human gastric carcinoma cell line (Ohgushi et al. 2005) and a B-cell line (Wildey et al. 2003).

Interestingly, TGF-β inhibits the expression of survivin as well as the activity of Akt in colon cancer cells, leading to apoptosis (Wang et al. 2008). It has been shown that Smad3 over-expression protects liver from HCC by promoting pro-apoptotic activity through TGF-β signaling and activation of p38MAPK (Yang et al. 2006b). Moreover, Yang and collaborators, showed that TGF-β via activation of Smad2 and Smad3, and in concert with the transcription factors Rb and E2F4, suppresses the prosurvival protein survivin in prostate epithelial cells (Yang et al. 2008a). In prostate epithelial cells, the prosurvival hormone dihydrotestosterone was found to suppress TβR-II expression (Song et al. 2006); this may explain the positive effects of androgens on proliferation and survival of prostate epithelial cells. Activation of p38 MAPK was shown to be essential to TGF-β-induced apoptosis in mammary epithelial cell (Yu et al. 2002), and B cells (Schrantz et al. 2001), but not in hepatocytes (Herrera et al. 2001c).

Bmf is another BH3-only protein implicated in TGF-β-induced apoptosis (Ramjaun et al. 2007) which plays a role in regulating the growth and survival of B cells (Wildey et al. 2003) and CLL cells (Morales et al. 2004). TGF-β transcriptionally regulates its expression through a ROS (Reactive Oxigen Species)-dependent mechanism (Ramjaun et al. 2007). In previous results of the group we suggested that the ROS producing system involved in this process is NOX4 (NAPDH Oxidase-4), which is required, at least, for up-regulation of the proapoptotic Bmf and Bim. In the case of Bim, regulation occurs at a post-transcriptional level (Caja et al. 2009), as it's been suggested by a mechanism involving Smad-3–dependent expression of the MAPK phosphatase MKP2 (Ramesh et al. 2008).

It's well known that Bcl-xL blocks TGF-β1-induced apoptosis by inhibiting cytochrome c release (Chipuk et al. 2001) and its induction together with repression of Bid (Ruan et al. 2010) or rapid stimulation of Mcl-1 (Gingery et al. 2008) promotes osteoclast survival. TGF-β potently induces apoptosis in Burkitt's lymphoma cell lines and in explanted primary human B lymphocytes by regulation of Bik and Bcl-xL and autocrine TGF-β signalling through ALK5 contributes to the default apoptotic programme in normal human centroblasts undergoing spontaneous apoptosis (Spender et al. 2009).

In the case of Bcl-2, it has been identified a protective role for TGF-β2 in osteoblast apoptosis induced by mechanical unloading via the $\alpha_5\beta_1$/PI3K/Akt signaling cascade and downstream Bcl-2 and phospho-Bad survival proteins (Dufour et al. 2008). In human lens epithelial cells the downregulation of Bcl-2 by TGF-β induces apoptosis without Caspase-3 activation neither poly (ADP-ribose) polymerase (PARP) activation (Lee et al. 2002).

ROS have been considered for years as toxic molecules generated as by-products during aerobic respiration, and implicated in different pathologic processes, such as inflammation or aging (Fialkow et al. 2007, Cecarini et al. 2007, Lenaz et al. 2000). However, ROS also function as signaling molecules at physiological concentrations mediating numerous biological processes and numerous cytokines and growth factors

have been described to generate ROS to transmit information upon binding to their receptors (Genestra 2007, Thannickal and Fanburg 2000). Previous results from our group in fetal rat hepatocytes showed that TGF-β induces cell death through a mechanism dependent on ROS production (Sanchez et al. 1996). ROS are responsible for the execution of the mitochondrial pathway of apoptosis (Herrera et al. 2001a), at least in part, through modulation of different members of the Bcl-2 family (Herrera et al. 2001b, Ramjaun et al. 2007, Kang et al. 2007). TGF-β-induced ROS have two main sources: one mitochondrial, through down-regulation of antioxidant genes (Franklin et al. 2003, Herrera et al. 2004); another one extra-mitochondrial, through NADPH oxidase (NOX) activation (Herrera et al. 2004). ROS production is blocked by the epidermal growth factor (EGF), which rescues hepatocytes from TGF-β induced-apoptosis (Carmona-Cuenca et al. 2006, Fabregat et al. 2000). NOX isoforms signals seem to be tissue and stimuli-specific. On the one hand, NOX4 is involved in apoptosis in human tissue- and stimuli-specific. On the one hand, NOX4 is involved in apoptosis in human aortic smooth muscle cells (Sturrock et al. 2006), leukemia cells (McKallip et al. 2006) and macrophages (Palozza et al. 2007), but it acts as a pro-survival signal for pancreatic cells against different stimuli (Vaquero et al. 2004, Edderkaoui et al. 2005). Previously we have reported that TGF-β induced NOX4 up-regulation and cell death in fetal hepatocytes, this effect being impaired by EGF addition (Carmona-Cuenca et al. 2006). In FaO cells, which show overactivation of the EGFR pathway (Caja et al. 2007), NOX4 is not efficiently induced by TGF-β and only when we inhibit the EGF receptor an up-regulation of NOX4 is detected at mRNA and protein level (Sancho et al. 2009). On the other hand, NOX1 is related to apoptosis in different tissues (Pantano et al. 2007) and cell lines (Yu et al. 2006, Lee et al. 2006d, Kim et al. 2007); but it is also reported to be implicated in cell survival in epithelial cells (Kobayashi et al. 2004, Morazzani et al. 2004) and it is overexpressed in colon adenoma and adenocarcinomas (Fukuyama et al. 2005). Our group have demonstrate in FaO rat hepatoma cells that NOX1 might control autocrine cell growth of liver tumor cells through regulation of the EGFR pathway and its knockdown increases caspase-3 activity and cell death, promoting more efficiently the pro-apoptotic mechanism induced by TGF-β when the EGFR is inhibited (Sancho et al. 2009, Sancho and Fabregat 2010).

The final decision to execute the apoptotic program is the result of the integration of all ongoing signaling within the cell and is likely to be determined in a cell-context-dependent manner (Hanahan and Weinberg 2011, Heldin et al. 2009).

5.7. TGF-β and EMT

Members of the transforming growth factor-β (TGF-β) superfamily initiate and maintain EMT in a variety of biological systems and pathophysiological conditions by activating major signaling pathways and transcriptional regulators (Bierie and Moses 2006, Zavadil and Bottinger 2005). Consistent with their binding to the same receptor complexes,

TGF-β1, TGF-β2 and TGF-β3 share the capacity to induce EMT in epithelial cells (Miettinen et al. 1994, Piek et al. 1999, Valcourt et al. 2005).

In cardiogenesis, TGF-β has been shown to play a key role in the EMT that occurs in the atrioventricular canal and the outflow tract region (Nakajima et al. 2000). In mice, TGF-β1 null embryos present severe cardiac abnormalities including defective atrioventricular junction. Moreover, TGF-β2-deficient mice present atrioventricular and outflow tract defects. Finally, blocking TGF-β activity using antibodies or antisense oligonucleotides inhibits EMT in chicken atrioventricular explants and TGF-β2-induced EMT in mouse explant cultures (Mercado-Pimentel and Runyan 2007, Azhar et al. 2003). In the other hand, TGF-β3 null mice present a cleft palate, resulting from the lack of fusion of the two palatal shelves (Nawshad et al. 2004).

Several lines of evidence implicate increased TGF-β signaling as a key effector of EMT in cancer progression and metastasis. Chemical carcinogenesis studies *in vivo* showed that transgenic expression of activated TGF-β1 correlates with the conversion of squamous into more invasive spindle cell carcinomas (Cui et al. 1996). Cancer cells often increase their production of active TGF-β, which not only triggers EMT and allows the cells to become invasive, but also enhance angiogenesis in close proximity to the tumor microenvironment, providing an exit route for migratory mesenchymal cells (Derynck et al. 2001). Focussing in liver, different studies have described EMT processes in hepatocytes and liver cancer cells (Sanchez et al. 1999, Rossmanith and Schulte-Hermann 2001, Valdes et al. 2002, del Castillo et al. 2006, Caja et al. 2007, Bertran et al. 2009, Franco et al. 2010).

The EMT process also occurs after tissue injury and contributes to organ fibrosis. Abnormally high levels of TGF-β are expressed in renal fibrotic sites of patients with kidney diseases. Moreover, transgenic mice with increased expression of TGF-β1 develop renal fibrosis (Schnaper et al. 2003). TGF-β has also been shown to play a key role in pulmonary and hepatic fibrosis (Willis and Borok 2007, Gressner et al. 2002). Finally, the recently described endothelial to mesenchymal transition may contribute to TGF-β-induced cardiac fibrosis (Zeisberg et al. 2007a).

Dominant negative interference with the TGF-β type II receptor function reverses EMT in colon cancer cells in culture and inhibits EMT in skin and mammary cancer models *in vivo* (Oft et al. 1998, Portella et al. 1998). In agreement, expression of an activated version of the receptors ALK-5 or ALK-4 recapitulates TGF-β-induced EMT in NMuMG cells (Valcourt et al. 2005, Piek et al. 1999), while dominant negative forms of either type I receptor block TGF-β-induced EMT (Valcourt et al. 2005).

Various studies have explored the roles of TGF-β-activated Smads in EMT. The TGFβ–IKKα–Smad signaling pathway induces transcription of the genes encoding Snai1 and Snai2 in pancreas liver cells (Brandl et al. 2010). The transcription factor c-Myc binds to Smads and induces Snail1 expression in response to TGF-β (Smith et al. 2009) and it's been described that a Snail1-Smad3/4 transcriptional repressor complex promotes TGF-

β mediated EMT (Vincent et al. 2009). Increased expression of Smad2 or Smad3 with Smad4 induces EMT, or enhances the induction of EMT by the activated form of TβRI in NMuMG cells (Piek et al. 1999, Valcourt et al. 2005), whereas expression of dominant negative versions of Smad2 or Smad3 blocks TGF-β-induced EMT in this cell system (Valcourt et al. 2005). Consistent with the pivotal role of Smad3 in EMT, renal tubular epithelial cells deficient in Smad3 fail to undergo EMT in response to TGF-β or mechanical stress (Sato et al. 2003), and keratinocytes derived from $Smad3^{-/-}$ mice show reduced migration in response to TGF-β (Ashcroft et al. 1999). Loss of Smad2 in keratinocytes promotes EMT and accelerates skin tumor formation. This has been explained by increased binding of the Smad3/4 complex to the promoter of the Snail gene and by increased Snail expression in the absence of Smad2, thus enhancing the progression of EMT (Hoot et al. 2008). Similarly, $Smad2^{-/-}$ hepatocytes appear mesenchymal and migrate faster than wild-type cells, while $Smad3^{-/-}$ hepatocytes retain their epithelial characteristics (Ju et al. 2006), suggesting that Smad3 may promote EMT *in vivo* (Oft et al. 2002). Smad4 is indispensable for EMT. RNA interference-mediated knockdown of Smad4 expression or expression of a dominant negative mutant of Smad4 results in preserved E-cadherin expression (Deckers et al. 2006, Valcourt et al. 2005, Takano et al. 2007, Kaimori et al. 2007). Smad6 controls the timing and extent of EMT during cardiac valve formation (Desgrosellier et al. 2005), while increased expression of Smad7 blocks TGF-β induced EMT in multiple tissues (Dooley et al. 2008, Xu et al. 2007, Valcourt et al. 2005, Zavadil and Bottinger 2005, Saika et al. 2004).

Focusing in hepatocytes, TGF-β has an essential role in the EMT of adult hepatocytes to fibroblastoid cells and the expression of Smad7 in hepatocytes of transgenic mice attenuated TGF-β signaling and EMT (Dooley et al. 2008). Similar results were obtained in further studies by employing immortalized murine AML-12 hepatocytes which show TGF-β-dependent EMT that is associated with activation of Smad2/3 signaling and collagen I synthesis as well as induction of Snail (Kaimori et al. 2007). Further studies revealed that hepatocytes isolated from cirrhotic livers, which are exposed to sustained TGF-β levels in vivo, differ in their phenotype from hepatocytes of healthy mouse livers by showing elongated, fibroblastoid cells expressing Vimentin and collagen I (Nitta et al. 2008). Smad2 is required for stable epithelial phenotype of primary hepatocytes *in vitro* however the relevance of this function *in vivo* remains to be determined (Ju et al. 2006). However, Smad3 is required for TGF-β induced EMT in vitro in primary hepatocytes as previously reported (Sato et al. 2007, Zavadil et al. 2004), although there's also been studied in the literature that TGF-β-induced EMT required both Smad2 and Smad3 signaling (Valcourt et al. 2005).

TGF-β cooperates with signalling pathways, such as Ras and Wnt to induce EMT (Massague 2008, Nawshad et al. 2005) and studies of EMT kinetics revealed that the induction phase of EMT involves the crosstalk between TGF-β and MAPK, and showed that the maintenance phase depends on the additional activation of PI3K/Akt (Fischer

et al. 2005). The Erk-MAPK pathway is activated by a number of growth factors, including TGFβ and oncogenic Ras, that are frequently upregulated in cancers (Dhillon et al. 2007). Activation of the Ras signaling pathway promotes the TGF-β-induced epithelial-mesenchymal transition (EMT) in mammary epithelial cells (Lindley and Briegel 2010). Increased Ras/Erk MAPK signaling enhances TGF-β-induced EMT, as apparent by the morphological changes and downregulation of E-cadherin expression (Uttamsingh et al. 2008, Grande et al. 2002, Lehmann et al. 2000) and blocking the kinase function of MEK1/2 TGF-β-induced EMT is inhibited (Xie et al. 2004).

TGF-β has been found to induce activation of Rho, Rac and Cdc42 in different cell systems, however, most studies have focused on the role of RhoA in TGF-β-induced EMT (Xu et al. 2009). The activation of RhoA in response to TGF-β in turn results in activation of ROCK, which induces the formation of actin stress fibers (Pellegrin and Mellor 2007). Moreover, TGF-β can activate LIM kinase, a downstream target of ROCK, which inactivates the actin-depolymerizing factor cofilin. Downregulation of LIM kinase expression using siRNA inhibits TGF-β-induced actin reorganization in fibroblasts (Vardouli et al. 2005). Activation of the PI3 kinase/Akt pathway by TGF-β plays a major role in EMT. Inhibitors of PI3 kinase and Akt, or a dominant negative form of Akt, were found to inhibit TGF-β-induced morphological transition, α-smooth muscle actin expression and E-Cadherin downregulation (Kattla et al. 2008, Bakin et al. 2000).

It is also well documented that the activation of p38 by TGF-β is involved in apoptosis and the EMT process (Heldin et al. 2009, Sorrentino et al. 2008, Yamashita et al. 2008a, Zhang 2009). Similar to its role in TGF-β-induced apoptosis, the TRAF6-TAK1-JNK/p38 pathway also plays a very important role in TGF-β-induced EMT. Inhibiting p38 activity impairs TGF-β-mediated changes in cell shape and reorganization of the actin cytoskeleton (Yu et al. 2002, Bakin et al. 2002). Knocking down TRAF6 expression also inhibits TGF-β-mediated EMT (Yamashita et al. 2008a).

5.8. TGF-β and tumor promotion

TGF-β provides a dual role in hepatocarcinogenesis since it triggers cell cycle arrest and apoptosis in the healthy liver and during tumor initiation, and in contrast, is capable to control dedifferentiation and spreading of neoplastic hepatocytes by induction of EMT (Fischer et al. 2005). It has been described that TRAF6 regulates the subcellular localization of TβRI by Lys63-dependent polyubiquitination and this can explain the capability of TGFβ to both act as a tumour suppressor and a promoter (Mu et al. 2011). Cells that survive to TGF-β apoptotic effect (Murillo et al. 2005, Caja et al. 2007) later induce epithelial-to-mesenchymal transition, a process that mediates cell migration and survival (Heldin et al. 2009, Valdes et al. 2002). Interestingly, liver tumors expressing late TGF-β–responsive genes (antiapoptotic and metastatic) display a higher invasive

phenotype and increased tumor recurrence when compared with those that show an early TGF-β signature (Coulouarn et al. 2008).

Many studies have identified the overexpression of TGF-β1 in various types of human cancer. Since the overexpression of TGF-β1 correlates with tumor progression, metastasis, angiogenesis and poor prognostic outcome (Bierie and Moses 2006, Levy and Hill 2006), the inhibition of TGF-β pathway has been targeted in therapeutic strategies in cancer. For example, soluble TGF-β type II receptor (TGF-βsRII) inhibits the action of TGF-β by binding to TGF-β1 and TGF-β3 with high affinity, and the administration of a recombinant TGF-βsRII protein significantly inhibits tumor growth and metastasis (Muraoka et al. 2002). Bone morphogenic protein-7 (BMP-7) is a member of the TGF-β superfamily, and is also a novel TGF-β inhibitor. BMP-7 binds and activates BMP type II receptor (BMP-RII) that subsequently form complex with BMP receptor type IA (BMPR-IA). The receptors activated by BMP-7 phosphorylate Smad1, 5 and 8, which counteract Smad2/3 phosphorylation by TGF-β, and antagonize against EMT (Inagaki and Okazaki 2007). Indeed, the administration of BMP-7 has been shown to reduce metastatic capability of breast cancer (Buijs et al. 2007).

The loss of a single copy of the gene encoding for the TGFβ type I receptor occurs in colorectal cancer patients. In a mouse model of type I receptor haploinsufficiency it's shown a weaker Smad2/3 activation and overproliferation of the mutant colonic epithelium, thus contributing to the progression of colorectal adenocarcinoma in these mice (Zeng et al. 2009). The same mechanism has been shown in head-and-neck squamous cell carcinomas in mice carrying an inducible and tissue-specific knockout of the TGF-β type I receptor (Bian et al. 2009). In these squamous carcinomas, inactivation of Smad2/3 signaling is correlated with strong activation of PI3K/Akt signaling that explains tumor overproliferation. The TGF-β inactivation-PI3K hyperactivation paradigm is of general importance in cancer development, as recently exemplified by studies in mice with prostate specific or pancreas-specific deletion of the Smad4 gene (Ding et al. 2011, Xu et al. 2010). A key synergistic factor that helps tumorigenesis in addition to the loss of Smad4 is the loss of the PTEN (phosphatase and tensin homolog) phosphatase that leads to abnormal activation of the PI3K pathway. The phosphorylation status of TβRI regulates its activity. The phosphatase PP2a is recruited to active TβRI to dephosphorylate it and thereby subsequently prevent further signaling. Interestingly, the decreased recruitment of PP2a to the activated TβRI in prostate cancer cells, compared with normal prostate epithelial cells, leads to increased TGF-β signaling in a manner dependent on Erk-MAPK (Yu et al. 2010). The elevated signals from activated TβRI have also been found to increase the autocrine production of TGF-β, resulting in a vicious cycle promoting the growth of tumor cells

Genetic inactivation of Smad signaling occurs in human cancer as revised in (Heldin and Moustakas 2011), for instance about 50% of pancreatic carcinoma show loss of Smad4 (Hahn et al. 1996). However, a more general phenomenon in diverse human cancers is the inactivation of the tumor suppressor function of Smad signaling by several

oncogenic signals. Many oncoproteins directly interact with or post-translationally modify the Smads thereby interfering with the Smad function. Accordingly, Ras-induced Erk MAP kinase signaling destabilizes Smad3 resulting in a significant loss of growth inhibitory activity of the pathway (Daly et al. 2010). In adult T-cell lymphoma, the transcriptional repressor zinc-finger E-box binding homeobox 1 (ZEB1), which binds to Smad2/Smad3 and enhances their transcriptional activity, is downregulated, whereas the inhibitory Smad7 is upregulated, thus providing a double signal that inactivates Smad signaling during the progression of this hematologic malignancy (Nakahata et al. 2010).

Different conditions in the tumor microenvironment and differential regulation of Smad2 and Smad3 can provide the signal for the switch in the role of TGF-β in cancer progression. The protein phosphatase PP2A preferentially dephosphorylates Smad3 and thus inactivates it, while leaving the Smad2 phosphorylation and function intact under the hypoxic conditions that are prevalent during tumor progression (Heikkinen et al. 2010b). Via this mechanism, antiproliferative responses such as p15 gene regulation are compromised because of the suboptimal function of Smad3, whereas other TGF-β/Smad responses remain intact and could possibly even be enhanced. This paradigm of the differential action of Smad2 and Smad3 is of major importance in TGF-β signaling during cancer progression and its importance was also highlighted in the previous section on EMT and cancer progression. Under normoxic conditions, the forced expression of Smad7 inhibits the invasive behavior of human cancer cells. In contrast, during hypoxia, the transcription factor HIF1α induces the expression of Smad7, which contributes to malignant cell invasiveness (Heikkinen et al. 2010a). Even non-tumorigenic human keratinocytes can become invasive when exposed to hypoxia and TGF-β and the suppression of the TGF-β-induced Smad7 could rescue this phenotype. In a different tumor model, colorectal cancer, forced Smad7 expression suppresses TGF-β-mediated anti-proliferative and pro-apoptotic responses, thus promoting tumorigenesis. Furthermore, Smad7 also promotes liver-specific metastasis of the colorectal tumor cells; this is correlated with active but subverted TGF-β signaling in the metastatic lesions (Halder et al. 2008). Studies of a mouse model with liver-specific knockout of the Smad7 gene have revealed that these otherwise healthy mice exhibit liver dysfunction and are prone to alcohol-induced tissue fibrosis, whereas exposure to TGF-β leads to accelerated EMT in the knockout hepatocytes (Zhu et al. 2011). Smad7 it's been shown to affect Wnt signaling though binding β-catenin, a key player in the oncogenic Wnt signaling pathway, and it's activated in various forms of cancer. TGFβ-induced accumulation of β-catenin in prostate cancer cells results in its nuclear translocation and apoptosis in a Smad7- and p38 MAPK-dependent manner (Edlund et al. 2005), whereas in keratinocytes, Smad7 has been found to have an opposite effect on β-catenin, as Smad7 recruitment of Smurf2 results in the degradation of β-catenin (Han et al. 2006).

6. Molecular mechanism of liver cancer

6.1. Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) accounts for more than five percent of all cancer cases and is the fifth leading cause of cancer mortality worldwide (El-Serag and Rudolph 2007, Parkin et al. 2005, Sherman 2005, Kensler et al. 2003). Although HCC frequency is highest in Asia and sub-Saharan Africa, the incidence and mortality rates are increasing in the United States in recent years and are anticipated to double over the next decade (El-Serag 2004).

The etiology of HCC includes major risk factors such as viral infection with hepatitis B or C (HBV, HCV), dietary exposure to fungal aflatoxin or alcohol intoxication (Farazi and DePinho 2006). Independent of the carcinogenic insult, chronic hepatitis and cirrhosis resulting from inflammation and fibrosis are present in almost eighty percent of HCC cases worldwide (Kensler et al. 2003, Friedman 2008). Additional etiological factors underlying fibrosis and cirrhosis such as hereditary hemochromatosis or non-alcoholic fatty liver disorders have also a potential impact in the development of HCC (Jou et al. 2008, Wallace and Subramaniam 2009). The cirrhotic lesions that commonly precede dysplastic HCC-like foci and nodules are prone to acquire initial genomic alterations which further accumulate during hepatocarcinogenesis (Teufel et al. 2007, Villanueva et al. 2007). About 80% of HCC patients are diagnosed at an advanced stage of disease due to the lack of symptoms during early stages of HCC and the rapid progression of disease (Sun and Sarna 2008).

As revised by van Zijl and collaborators (van Zijl et al. 2009), in the pathogenesis of HCC, multiple and diverse mechanisms have been reported to cause the aberrant proliferation and dedifferentiation of hepatocytes which lead to the subsequent development of malignant neoplasia. Most frequently, (i) inactivation of tumor suppressors such as p53, retinoblastoma or the CDKN2A-encoded proteins p14ARF and p16Ink4A, (ii) overexpression of cyclin D1/Cdk4, insulin-like growth factor-II or c-MET as well as (iii) activation of the Ras/mitogen activated protein kinase (MAPK), transforming growth factor (TGF)-β signaling or Wnt/β-catenin signaling have been described as critical events in HCC (El-Serag and Rudolph 2007, Teufel et al. 2007, Villanueva et al. 2007, Breuhahn et al. 2006, Tannapfel et al. 2001).

Despite the current improvements in treatment (Llovet and Bruix 2008) and diagnostics, only 30–40% of patients with HCC are eligible for curative therapy (Llovet et al. 2003). In general, liver resection and orthotopic liver transplantation are considered as the only curative treatments of HCC, and the prognosis of HCC is poor when the tumor burden cannot be surgically removed (Llovet and Bruix 2008). Despite major efforts in past decades, therapeutic options to interfere with HCC progression are very limited and novel therapeutic strategies for efficient treatment are needed.

The most important parameter to select patients for OLT (Orthotopic liver transplantation) is still the size and number of tumors, since the Milan criteria defined in 1996 demand that only HCC patients with one or three nodules measuring 5 or up to 3 cm in diameter, respectively, are recommended for OLT (Mazzaferro et al. 1996). However, these Milan criteria are a continuing matter of debate, particularly as new clinicopathological and prognostic markers allowing a better selection for OLT have been defined, giving also those patients an access to transplantation who exceed the Milan criteria (Ishizaki and Kawasaki 2008).

Insights into the molecular pathogenesis of HCC have revealed a substantial heterogeneity of the malignancy (Lee and Thorgeirsson 2004). In most instances, HCC develops in a liver compromised by chronic hepatitis and/or cirrhosis (Grisham 1997). There is extensive evidence that under these conditions of tissue injury the normally quiescent adult liver stem cells are activated (Hsia et al. 1992) and thus become a potential target cell population in liver cancer (Komuta et al. 2008, Libbrecht et al. 2002, Theise et al. 1999). This notion is supported by the data demonstrating a progressive upregulation of hepatic progenitor cell (HPC) markers in cirrhosis (Tanaka et al. 2005) as well as in dysplastic nodules in human liver (Libbrecht et al. 2000) and adenoma (Libbrecht et al. 2001). In rodents, hepatic stem/progenitor cell origin of HCC has been also postulated (Maronpot et al. 1986, Sell and Dunsford 1989).

Several studies have suggested the stem/progenitor cell origin of liver cancers including HCC (Lee et al. 2006c, Yamashita et al. 2009) and HCC that expressed progenitor cell/ductular markers, such as Cytokeratin(CK) 7 and CK19, presented a more aggressive clinical course and more frequent and rapid recurrence of disease after surgical treatment (Durnez et al. 2006, Uenishi et al. 2003). CK19, a marker of both cholangiocytes (bile duct epithelial cells) and oval cells (bipotential hepatic progenitors) is an important example, since its enhanced expression correlated with a higher recurrence rate after OLT (Durnez et al. 2006).

Furthermore, Fiorentino and collaborators proposed that a combination of low membranous E-cadherin, high proliferative MIB-1 index and nuclear β-catenin accumulation correlated with elevated relapse of HCC after OLT. However, only six of 83 patients with high nuclear β-catenin were reported in their study, a much lower percentage than usually observed (Lee et al. 2006a). Fiorentino and collaborators found 58.6% of 133 HCC patient samples with high or medium nuclear β-catenin expression, which is in good agreement with previous investigations (Lee et al. 2006a) and they suggest that high levels of nuclear β-catenin-positive tumor cells could provide an excellent prognostic marker for the relapse of HCC (Fiorentino et al. 2004).

6.2. Cholangiocarcinoma (CC)

Cholangiocarcinoma (CC) arises from the ductal epithelium of the bile duct tree and is classified anatomically into intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC). The incidence and mortality rates of cholangiocarcinoma, especially those of ICC, are increasing worldwide (Khan et al. 2003).

As revised in Sempoux and colleagues (Sempoux et al. 2011b), the incidence of these tumors is different, ECCs affect men more than women with a peak age in the sixth or seventh decade and are often detected at an early stage with signs of biliary obstruction. In contrast, ICCs typically occur in patients in the fifth or sixth decade of life, with no frank sex predilection and patients remain asymptomatic for a longer period (Aishima et al. 2007, Malhi and Gores 2006, Khan et al. 2005a, Okuda et al. 1977, Klatskin 1965). Recent epidemiologic studies suggest chronic hepatitis C virus (HCV) infection as a major risk factor for ICC (Yamamoto et al. 2004, Okuda et al. 2002a).

Cholangiocarcinoma is known to have a bad response to chemotherapeutic treatment (Shimoda and Kubota 2007) and complete resection is the only way to cure the disease at present. Moreover, because cholangiocarcinoma is difficult to diagnose at an early stage and extends diffusely, most patients have unresectable disease at clinical presentation, and prognosis is very poor (5-year survival is 0–40% even in resected cases) (Khan et al. 2003, Sirica 2005).

Recently, a number of experimental treatment options have come to the forefront. The most promising of these appears to be a multiple kinase inhibitor, Sorafenib (Wilhelm et al. 2008). Recently, Sorafenib was shown to display significant tumor suppression in a rodent model of cholangiocarcinoma (Blechacz et al. 2009) and is currently undergoing phase II trials (Bengala et al. 2010). Another strategy to improve the treatment options of cholangiocarcinoma is to increase the sensitivity of cholangiocarcinoma to common chemotherapeutic agents (Francis et al. 2010).

The mechanisms involved in cholangiocarcinogenesis are highly variable, reflecting the differences between ICC and ECC, and the variations in geographic distribution and risk factors (Blechacz and Gores 2008, Malhi and Gores 2006, Shaib and El-Serag 2004). As revised in Sempoux and colleagues (Sempoux et al. 2011b), neoplastic transformation of the cholangiocytes appears to be driven by chronic inflammation and bile stasis within the biliary tree, following a multistep process initiated by the release of growth-promoting factors, such as tumor growth factor-beta (TGF-β) and cytokines (Blechacz and Gores 2008, Khan et al. 2008, Malhi and Gores 2006, Sirica 2005, Berthiaume and Wands 2004, Nakanuma et al. 2003, Okuda et al. 2002b). Cyclooxygenase 2 upregulation, EGFR phosphorylation, and hepatocyte growth factor overexpression, as well as a variety of oncogenic mutations have also been identified. Mutations of Kras and nuclear accumulation of p53 protein have been reported with some frequency (Khan et al. 2005b). Patients with high α-smooth muscle actin expression in stromal

cells were shown to experience worse outcomes, shedding light on the possible influence of myofibroblasts on ICC progression (Okabe et al. 2009a).

Cytokeratin 19 is an important structural component of the epithelial cytoskeleton, generally expressed on cholangiocytes and ICC cells (Corcelle et al. 2006, Dobashi et al. 2000) and together with Cytokeratin 7 are good markers of biliary epithelial differenciation and are found in 90% of cholangiocarcinomas (Maeda et al. 1995). By performing gene expression profiling of human CC, Woo and collaborators (Woo et al. 2010) found wellknown biomarkers for CC or hepatic progenitor cells such as CK19, EpCAM and CD133 were identified. Also, known CC biomarkers such as CEACAM6 (Ieta et al. 2006), MUC1 (Higashi et al. 1999), and CLDN4 (Nishino et al. 2008) were identified indicating the usefulness of the CC signature as novel differential biomarkers for CC. There's also been suggested the stem/progenitor cell origin of CC (Nomoto et al. 2006) among other liver cancers and such heterogeneous differentiation status of cellular origin suggests a phenotypic overlap among them.

6.2.1. Combined hepatocellular-cholangiocarcinoma (CHC)

Hepatocellular cholangiocarcinoma (CHC), is a rare form of primary liver cancer that was initially described by Allen and Lisa in 1949 (Allen and Lisa 1949) and includes the composition and characteristics of HCC and CC coexisting in the same tumor or liver. The incidence of CHC among primary liver cancers is about 1.0–4.7% (Ng et al. 1998, Aoki et al. 1993).

According to the World Health Organization classification, the histopathological definition of CHC, as well as its diagnosis, is based on criteria that require unequivocal elements of both HCC and CC (Ishak et al. 1994).However, some CHC tumours exhibit separable areas between HCC and CC and many show intermediate features in most or all of the lesions, which makes classification based on these criteria challenging. Goodman and colleagues (Goodman et al. 1985),Taguchi and collaborators (Taguchi et al. 1996) and Libbrecht (Libbrecht 2006) have identified different criteria for the pathomorphological diagnosis of CHC, and classified this disease into three categories, respectively: type I, collision tumours and double cancers, where the HCC and CC components are either completely separated or sharply demarcated; type II, tumours with contiguous, independent masses of HCC and CC, separated by an intervening area of transition; and type III, tumours consisting entirely of transitional areas, containing only very limited, ambiguous hepatocellular and cholangiocellular components.

The diagnosis of combined HCC/ICC requires unequivocal histologic presence of both hepatocellular and cholangiocellular elements intimately mixed within the same tumor. As revised in Sempoux and collaborators, the hepatocellular characteristics are a trabecular pattern of growth and bile production whereas the cholangiocellular characteristics are a glandular pattern and mucin production. In some tumors, PAS

reaction may help in demonstrating mucin production in the ICC component. CK7 and CK19 immunostains are positive in the cholangiocellular component, while glypican-3, α-fetoprotein (AFP), HepPar1, and canalicular staining pattern with polyclonal CEA are useful in identifying the HCC component. These HCC markers are often negative in poorly differentiated HCC and the lack of staining with CK7 and CK19 might be sufficient to recognize the HCC component (Sempoux et al. 2011b). Glypican-3, a developed marker of HCC (Capurro et al. 2003) although it's also expressed in hepatoblastomas (Zynger et al. 2008), is a helpful complementary marker because contrary to other hepatocyte markers such as HepPar1, it is more frequently expressed in less differentiated HCC (Shafizadeh et al. 2008). It should also be kept in mind that only one of the components may be present in a biopsy specimen and that HCC may also express CK7 and CK19 with some frequency.

CHC have been suggested to be derived from bipotential liver stem cells which can differentiate into either hepatic or biliary progenitor cells (Zhang et al. 2008, Komuta et al. 2008). This supports the hypothesis that CHC originates from hepatic stem or progenitor cells, which have the potential to differentiate into both hepatocytic and cholangiocytic lineages. The patients with CHC showed poor prognosis similar to patients with CC, suggesting the closer likeliness of CC and CHC (Cazals-Hatem et al. 2004, Woo et al. 2010).

6.2.2. Cholangiolocellular carcinoma (CLC)

Cholangiolocellular carcinoma (CLC) is a rare malignant primary liver tumor accounting for 1% of all primary liver cancer. Steiner and Higginson (Steiner and Higginson 1959) first defined this entity and categorized it as a subtype of cholangiocellular carcinoma (CC). It is frequently associated with HCV infections (Komuta et al. 2008).

According to its unique histological features, CLC is thought to originate from the ductules and/or canals of Hering where hepatic progenitor cells (HPCs) are located (Komuta et al. 2008). CLCs resemble cholangioles (bile ductules) and share HPC immunomarkers such as CK7, CK19, and N-CAM (Komuta et al. 2008, Kanamoto et al. 2008). As revised in Sempoux and colleagues (Sempoux et al. 2011b), the HPC origin of CLC is reinforced by the frequent coexistence of an HCC and/or a classic ICC component within CLC.

CLCs are white, solid, unencapsulated, usually solitary lesions characterized by proliferation of small cords of epithelial cells mimicking ductular reaction in an abundant fibrous stroma, with some features of ductal plate malformation (Steiner and Higginson 1959). The tumor cells show minimal atypia; hence, this lesion can be very difficult to differentiate from benign ductular reaction and from bile duct adenoma. The infiltrative growth pattern at the borders, the anastomosing architecture of the ductules, and the expression of p53 oncoprotein may be of help (Sempoux et al.

2011a). Although N-CAM is useful in differentiating CLC from classical well-differentiated ICCs, which commonly do not express this marker, BDA and ductular reaction (Gutgemann et al. 2006, Roskams et al. 1990) are also N-CAM positive. Therefore, this immunomarker is not helpful in differentiating CLC from these benign lesions. The strong resemblance with ductular reaction gave rise to other terminologies, perhaps more appropriately, such as ‘‘bile ductular carcinomas’’ or ‘‘ductular cholangiocarcinomas.’’(Nakanuma et al. 2008, Kozaka et al. 2007). In current practice, CLC is probably underrecognized and underdiagnosed, partly because when HCC and/or ICC areas are present, the CLC component is often ignored.

6.2.3. Cholangiocarcinoma-like HCC (CLHCC)

Recently it has been described by a single group a novel subtype of HCC, cholangiocarcinoma-like HCC (CLHCC). By performing gene expression profiling of human HCC (hepatocellular carcinoma), CHC (combined hepatocellular-cholangiocarcinoma), and CC (cholangiocarcinoma), they identified a novel HCC subtype, cholangiocarcinoma-like HCC (CLHCC), which expressed cholangiocarcinoma-like traits (CC signature). Similar to CC and CHC, CLHCC showed an aggressive phenotype with shorter recurrence-free and overall survival. In addition, they found that CLHCC coexpressed embryonic stem cell–like expression traits (ES signature) suggesting its derivation from bipotent hepatic progenitor cells. By comparing the expression of CC signature with previous ES-like, hepatoblast-like, or proliferation-related traits, they observed that the prognostic value of the CC signatures was independent of the expression of those signatures. In conclusion, they suggested that the acquisition of cholangiocarcinoma-like expression traits plays a critical role in the heterogeneous progression of HCC (Woo et al. 2010).

Lu and collaborators described a HCC-expressing cholangiocyte markers and, however they named it DPHCC (dual-phenotype HCC), it seems the same kind of tumor described by Woo and colleagues the previous year (CLHCC). They accounted for 10.1% of total HCCs they analyzed. These tumors expressed elevated serum α-fetoprotein and CA19-9 and they have done a correlation with the immunostaining of CK19. DPHCC has a highly aggressive behavior and worse postoperative prognosis than pure HCC and they recommend that CK19 be routinely used in the pathological diagnosis of HCC for screening DPHCC(Lu et al. 2011).

6.3. Hepatoblastoma (HB)

Hepatoblastoma (HB) is the most common primary malignant tumor of the liver in young children, accounting for approximately 1% of all childhood malignancies in Western countries (Mann et al. 1990).

Histologically, HBs are characterized by a diversity of epithelial and often mesenchymal patterns of differentiation (Weinberg and Finegold 1983), with some epithelial variants morphologically resembling stages of liver development. HBs with pure fetal (PF) epithelial morphology comprise about 15% of tumors. When fetal HB with low mitotic activity can be surgically resected it can be cured without chemotherapy (Weinberg and Finegold 1983) . All other HB subtypes have an aggressive phenotype requiring chemotherapy as well as surgical resection, and in some cases liver transplantation. The presence of a significant small-cell component in HB has been associated with particularly aggressive behavior and resistance to therapy (Haas et al. 2001).

A strong association between HB and extreme prematurity has also been documented (Buckley et al. 1989). Cytogenetic analysis (Schneider et al. 1997) has only been reported in a small number of cases, and there are only rare genomic and expression profiling studies available (Terracciano et al. 2003, Luo et al. 2006). Luo and colleagues found the genes IGF2 (insulin-like growth factor 2), Fibronectin, DLK1 (Delta-like 1 homolog), TGF-β1, MALAT1 (Metastasis associated lung adenocarcinoma) and MIG6 (Mitogen inducible gene 6) over-expressed in hepatoblastoma of childhood versus HCC but they not exclusive of HB. For instance, there are contradictory studies which found that IGF2 is also expressed in hepatocellular carcinoma (Kim and Lee 1997, Harris et al. 1998, Ma et al. 2011), Fibronectin is found to be expressed both in hepatocellular carcinoma (Torbenson et al. 2002, Gupta et al. 2006) and in cholangiocarcinoma (Chen et al. 2003), DLK1 is also found expressed in HCC (Huang et al. 2007, Yanai et al. 2010), TGF-β1 is expressed both in HCC (Giannelli et al. 2002, Bedossa et al. 1995, Ito et al. 1991) and CC (Benckert et al. 2003, Ohira et al. 2006). As a consequence, the overall molecular pathogenesis of HB remains poorly understood.

Hepatoblastomas, like other embryonal malignant tumors, are believed to arise from cellular populations that have not completed the process of differentiation. Their putative oncogenic mechanisms may involve inherent risks associated with the complex process of normal development (Maris and Denny 2002). Sakairi and collaborators (Sakairi et al. 2001b) found a multidirectional differentiation potential in mice hepatoblastoma induction, suggesting a possibility of de novo generation from undifferentiated stem cells. The same group analyzed an stablished cell line from a mouse hepatoblastoma and the evaluation of gene expression revealed that MHB-2 cells were positive for CK8/18 but negative for c-kit, CD34 (thymus cell antigen), Thy-1 and albumin on protein level although there were differences when analyzed by immunohistochemical staining of the HB in vivo or in situ hybridization. They conclude that the mouse HB have de-differentiated, bipotent, or biliary-like cell characteristics

and suggested that the mouse HB cells are closely like some sort of hepatic undifferentiated cells (Sakairi et al. 2007).

In rodents, hepatoblastomas (HBs) are occasionally observed in aged mice (Frith et al. 1994) and also can be induced by certain chemicals in some strains (Diwan et al. 1995). Mouse HBs are histologically similar to embryonal or small cell type tumors in man (Diwan et al. 1995). The origin is still unclear in mice as well as in humans, although several reports have suggested a derivation from the hepatocellular lineage (Calvert et al. 1995, Diwan et al. 1995).

A better understanding of the biological basis of HB and the differences between clinical subtypes may help in the development of biological markers for patient stratification and treatment strategies that could improve the outlook for some of these patients.

IV. BACKGROUND OF THE GROUP RELATED TO THE CURRENT phD WORK

The dual role played by TGF-β in tumorigenesis has been a topic of interest in our group. Over the last years we have been exploring the different pathways induced by TGF-β in hepatocytes and liver tumor cells.

We have shown that TGF-β inhibits hepatocyte growth, arresting cells in G1 phase of the cell cycle and down-regulating c-myc expression (Sanchez et al. 1995). Furthermore, TGF-β acts synergistically with the epidermal growth factor (EGF), promoting morphological changes related to differentiation of these cells (Sanchez et al. 1998). In addition to these functions and when used at higher concentrations, TGF-β induces cell death, through a mechanism dependent on reactive oxygen species (ROS) production (Sanchez et al. 1996). ROS are responsible for the execution of the mitochondrial pathway of apoptosis (Herrera et al. 2001a), at least in part, through modulation of different members of the Bcl-2 family (Herrera et al. 2001b). TGF-β-induced ROS have two main sources: one mitochondrial, through down-regulation of antioxidant genes, another one extra-mitochondrial, through NADPH oxidase activation (Herrera et al. 2004).

However, additionally to its capacity to induce apoptosis, TGF-β also induces anti-apoptotic signals in fetal hepatocytes and liver tumor cells (Valdes et al. 2004), through the expression of the epidermal growth factor receptor (EGFR) ligands transforming growth factor-alpha (TGF-α) and heparin-binding EGF-like growth factor (HB-EGF) and activation of the metaloprotease TACE/ADAM17 (TNF-α converting enzyme) responsible for their shedding (Murillo et al. 2005, Caja et al. 2007). ROS production is blocked by the EGF pathway, which rescues hepatocytes from TGF-β induced-apoptosis (Carmona-Cuenca et al. 2006, Fabregat et al. 2000).

Cells that survive to TGF-β-induced apoptotic effects undergo EMT (Sanchez et al. 1999, Valdes et al. 2002, Caja et al. 2007), cells presenting a fibroblastic appearance, an increased motility, and a replacement of the Cytokeratin network by Vimentin as the intermediate filament. The resulting cells show high levels of Vimentin, alpha-smooth muscle actin (α-SMA) and Snail expression and lack Cytokeratin 18 and E-Cadherin. We have also reported that EMT induces cell de-differentiation. Thus, fetal rat hepatocytes chronically treated with TGF-β show 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) , characteristic of terminally differentiated liver cells (Sanchez et al. 1999, Valdes et al. 2002) and, interestingly, EMT induces the expression of progenitor markers (Fabregat et al. 1996, Sanchez et al. 1999, Valdes et al. 2002, Caja et al. 2011a). In fact, TGF-β induces transdifferentiation of fetal hepatocytes to liver progenitors with capability of differentiating to both mature hepatocytes and cholangiocytes (del Castillo et al. 2008, Caja et al. 2011a).

Furthermore, the EMT process confers to the cells resistance to apoptosis, coincident with higher expression of Bcl-xL and basal activation of AKT (Valdes et al. 2002). Actually, there is no doubt that phosphatidylinositol 3-kinase (PI3K) pathway interferes

with the apoptotic effect of TGF-β in different cell types. We have also reported that the early activation of Akt can be related to the TGF-β ability of transactivating c-Src and EGF pathways in hepatocytes (Murillo et al. 2005) and mediates carcinogenesis by transactivation of the EGFR pathway in FaO rat hepatoma cells (Caja et al. 2007). The activation of c-Src by TGF-β is EGFR dependent and is required for full Akt phosphorylation and therefore cell survival in rat hepatocytes (Murillo et al. 2005). Indeed, EMT might confer resistance to TGF-β suppressor effects through the increase in the expression of EGFR ligands and activation of the EGFR pathway, which points out to a clear crosstalk between TGF-β and EGF signals in liver cells (del Castillo et al. 2006, Caja et al. 2007). In addition, preliminary data of our group in MDCK and melanoma cells had indicated that molecules involved in EMT, such as Snail and SPARC, might regulate proliferation and cell death (Vega et al. 2004) and that SPARC expression was regulated by Snail in MDCK cells (Moreno-Bueno et al. 2006, Olmeda et al. 2007). However, when this work started, we ignored whether Snail or SPARC might be related to the survival signals increased in the liver cells after undergoing EMT.

When this PhD project started, the group was very interested in better understanding the mechanisms responsible for the resistance to TGF-β-induced apoptosis in both non-tumoral hepatocytes and liver hepatoma cells. We also wanted to test the tumorigenic potential of hepatoma cells after its *in vitro* chronic treatment with TGF-β, in order to analyze the relevance of TGF-β-induced EMT in liver tumor progression.

V. AIMS

General Objective

TO IDENTIFY TGF-β REGULATED GENES IN HEPATIC CELLS THAT BEING INVOLVED IN CELL INVASION COULD ALSO PARTICIPATE IN THE CONTROL OF GROWTH, APOPTOSIS AND/OR DIFFERENTIATION.

Specific Objectives

1. Analysis of genes regulated by TGF-β and implicated in EMT that might participate in the apoptosis control in hepatocytes: role of Snail and SPARC.

2. Study the *in vivo* tumorigenesis of *in vitro* TGF-β-chronically-treated tumor liver cells: morphological and phenotypical analysis and apoptotic response.

VI. MATERIAL AND METHODS

1. Cell Culture

1.1. Cell lines: neonatal murine hepatocytes, Hep3B, SK-Hep-1, FaO

All cell lines were obtained from the European Collection of Cell Cultures (ECACC), except for the immortalized neonatal murine hepatocytes (MH) which were obtained as described previously (Gonzalez-Rodriguez et al. 2008). The hepatocyte cultures were maintained in DMEM medium, whereas the human liver carcinoma cell lines Hep3B (hepatocyte carcinoma) were maintained in MEM medium, and SK-Hep1 (liver adenocarcinoma) were grown in MEM supplemented with 1mM Sodium Pyruvate. The rat hepatoma FaO cells were grown in F12 Coon's Modified medium. All media were supplemented with 10 % (v/v) foetal bovine serum and cultures were maintained in a humidified atmosphere of 37°C, 5% CO_2. Further sub-confluent cultures (70-80%) were splited by trypsinization. For experiments, cells at 70% confluence were serum-starved during 8 to 12 hours before treatments, except neonatal murine hepatocytes which were starved only during 4 hours at 2% (v/v) of fetal bovine serum. After this time it is considered that cells lose the proliferative stimuli obtained from fetal bovine serum components.

1.2. Cristal Violet Staining

The number of attached cells after treatments was estimated by the crystal violet method. Briefly, cells were plated in 12 or 24-well plates respectively and cultured at the described conditions until they reached the experimental end-point. On the indicated days, media was removed and cells were washed twice with PBS and then stained using a solution of Crystal violet at 0.2 % (w/v) in 2% (v/v) ethanol for 30 minutes at room temperature (RT). Then, the staining solution was removed, and the wells were washed several times with PBS or distilled water until the excess staining that hadn't been incorporated into the cells was eliminated. The plate was air-dried, and the colorant dissolved using a 10%(w/v) SDS solution. Absorbance was measured at 595 nm in a spectrophotometric plate reader. The results were expressed as the percentage (+/- SD) of control cells at the experimental indicated time.

1.3. Duct formation assay (Matrigel assay)

This assay was carried out to analyze the formation of ductal structures by cells growing onto basement membrane (MatrigelTM). Briefly, cells were grown onto 12-well plates previously coated with Matrigel. The thick Matrigel layer was prepared by adding 250µL/well of 1:1 solution into the cold 12-well plate following the manufacturer's reccommendations. The plate was left at 37°C for 1.5 hours to stabilize the Matrigel coat and then cell were seeded on top in complete medium. After their attachment, the foetal bovine serum was removed. The cultures were incubated at the conditions described below and maintained for 24 - 48 hours under starvation conditions. Duct formation was visualized in a LEICA DM IL LED and representative images were acquired in a LEICA DFC 420C camera and edited in Adobe Photoshop CS.

2. Analysis of cell proliferation

2.1. Cell proliferation and cell death Analysis

Cell cycle profiles and sub-G1 analysis were performed by flow-cytometric analysis of propidium iodide-stained cells. Briefly, attached and unattached cells after treatments were collected together and then centrifuged at 2500 rpm for 5 min. The pellet was resuspended in 200 µL of PBS and added, drop by drop and then fixed with 500 µL of cold 100% Ethanol. At this point, samples could be stored at -20°C. To collect the fixed cells, samples were centrifuged at 2500 rpm for 5 minutes at 4°C. The pellet was air-dried and resuspended in 250µL of PBS containing 0.1 mg/mL of RNAse. Fixed cells were stained with propidium iodide (final concentration of 0.05% (w/v)) at 37^{o}C for 30 min. Cells were analysed for DNA content using a flow cytometer FACScan (Becton-Dickinson) at the Serveis Cientifico-Tecnics, Universitat de Barcelona. Data analysis was carried out using the software ModFit LTTM (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

Cells were incubated in 6-well plates or 60mm dishes until they reached the experimental end-point. Then they were scraped and both media and cells were collected in a 15mL tube, which was then centrifuged at 2500 rpm for 5 min. Indeed, this pellet contained those cells that were attached to the tissue culture dish and those that were dead and floating in the media. The pellet was resuspended in 30 to 100µL

lysis buffer, Table m.I. The solution was then transferred to an Eppendorf tube, which was incubated for 20 minutes on ice and vortexed every minute. After this time, the Eppendorfs were centrifuged at 13000 rpm during 10 minutes at 4°C. The supernatant was stored at -20 or -80°C until it was used.

To determine Caspase-3 Activity, first we determined the protein concentration by Bradford's method. Then a mix containing 20µg of protein (in a final volume of 25µL), 125µL of Buffer Reaction 2X (Table m.II) and 2µL of fluorogenic substrate for caspase-3, Ac-DEVD-AMC (BD Pharmigen) was 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 was measured using an exciting wavelength of 360nm and an emission wavelength of 440nm.

Table m.I. Caspase-3 Lysis Buffer	
Tris-HCl pH8	5mM
EDTA	20mM
Triton-X-100	0.5%

Table m.II. Caspase-3 Reaction Buffer 2X	
Hepes pH7.5	40mM
Glyrecol	20%
DTT	4mM

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 were usually represented as percentage of increase versus control.

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

The intracellular DNA content was analyzed by flow cytometry, as described in the section 2.1. 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 were plated on 2% gelatin-coated glass coverslips until they reached the experimental end-point. The monolayer was washed with PBS, cells were fixed with 4% paraformaldehyde in PBS for 20 min at RT, and were permeabilized 2 minutes with 0.1% Triton X-100-1% BSA. Primary antibodies, anti-Bax antibody 6A7 clone and anti-Bak G317-2 clone (BD Pharmingen) (1:50) were diluted in 1% BSA and incubated for 16 hours at 4°C in a humidity chamber. After several washes with PBS, the samples were incubated with fluorescent-conjugated secondary antibodies (1:200 for Alexa Fluor 488-

conjugated anti-mouse) for 1 hour at RT and embedded in Vectahield with DAPI (Vector Laboratories, Burlingame, CA). Cells were visualized in an Olympus BX-60 microscope or a Leica DMR 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 edited in Adobe Photoshop. Positive cells were counted and results were shown as percentage of positive cells relative to cell number.

3.4. Analysis of the percentage of apoptotic nuclei

Cells were plated on 2% gelatin-coated glass coverslips until they reached the experimental end-point. The monolayer was washed with PBS, cells were fixed with 4% paraformaldehyde in PBS for 20 min at RT and washed several times with PBS. Cells were embedded in Vectahield with DAPI (Vector Laboratories, Burlingame, CA) and were visualized in an Olympus BX-60 microscope or a Leica DMR microscope with the appropriate filters. Representative images were taken with a Spot 4.3 digital camera and edited in Adobe Photoshop. Apoptotic nuclei were quantified via those with a fragmented and/or pyknotic appearance and results were shown as percentage of apoptotic nuclei relative to total cell number.

3.5. Anoikis assay

The assay was carried out to analyse the cell survival under un-attached conditions (detachment-induced cell death). Briefly, cells in suspension were plated on 10 cm-plastic bacterial dishes in complete media and incubated at 37^{o}C for 48 hours. After this time, the number of cells attached to untreated plastic were stained with Crystal Violet and counted (25 random fields were counted under the microscope). Cells that did not attach to the bacteria dishes were recovered and then plated, at identical cell density, onto plastic cell culture dishes in complete medium and maintained for additional 48 hours. The number of attached cells was quantified by Crystal Violet, as described in the section 1.2. The results were expressed as percentage (+/- SD) of the absorbance relative to that of control cells.

4. Analysis of gene expression

4.1. RNA isolation and RT-PCR

RNeasy Mini Kit (Qiagen, Valencia, CA) was used for total RNA isolation. Reverse transcription was carried out using High Capacity Reverse Transcriptase kit (Applied

Biosystems, Foster City, CA, USA), with 500ng of total RNA from each sample for complementary DNA synthesis, following manufacturer's instructions.

4.2. Semi-quantitative PCR

Semi-quantitative PCR reactions were performed using 5 µl of 1:10 diluted cDNA, using a final concentration of 1 µM of mouse (m, mus musculus) or rat (r, rattus novergicus) specific primers (Table m.IV), dNTPS at 100 µM, 1.5 mM MgCl2 and 1 unit of Ecotaq (Ecogen). PCR reactions were carried out following the conditions shown in Table m.III.

Table m.III. PCR conditions

Phase	Temperature ^{o}C	Time	N^{o} 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	10 min	1

The obtained PCR products were 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.

Table m.IV. Primer sequences used in semi-quantitative PCR

Gene	Forward primer	Reverse primer	Tm ºC	Nº Cycles
r-Alb	5'CTGCCGATCTGCCCTCAATAGC 3'	5'GTGCCCACTCTTCCCAGGTTTCT 3'	58	25
m-Bcl2l1	5' CGTGGAAAGCGTAGACAAGG 3'	5' GAGCCCAGCAGAACTACACC 3'	60	24
m-Cdh1	5' TCCTGTCTTCAACCCGAGCACG 3'	5' TCCATGAACGTGTCCGGCTCTC 3'	55	30
r-Epcam	5' ACAAGGACACGGAGATCACG 3'	5' GCTCTCCGTTCACTCTCAGG 3'	59	35
r-Foxa2	5'GCACCTGAGTCCGAGTCTGAG 3'	5'GAGCTGAACCTGAGAAGCCTG 3'	63	30
m-Hbegf	5'CGGTGGTGCTGAAGCTCTTTC 3'	5'TGGTAACCAGGGAGGCAGTG 3'	59	30
r-Hnf4a	5'AGTACATCCCGGCCTTCTGTG 3'	5'GACCCTCCAAGCAGCATCTCC 3'	63	30
m-Kit	5' ACAAGAGGAGATCCGCAAGA 3'	5' GAGCTCCCAGAGGAAAATCC 3'	60	40
r-Kit	5'AGCAAGAGTTAACGATTCCGGAG 3'	5'CCAGAAAGGTGTAAGTGCCTCCT 3'	58	40
r-Krt7	5' TCTCGCTCCACTGCTTACCCG 3'	5' GGCCACACGAGGCCTTGATGA 3'	60	40
m-Krt18	5' CAAGATCATCGAAGACCTGAGGG CTCAGAT 3'	5' TACTTGTCCAGTTCCTCACGGTT CTTCTGA 3'	63	30
r-Krt19	5' TTGCGGACCAAGTTTGAGACAG 3'	5' CCGTGACCTCAGTCTTGTTTATC 3'	56	30
m-Mcl1	5' TTCTTGTAAGGACGAAGCGGG 3'	5' GCCAGCAGCACATTTCTGATG 3'	55	30
m,r-Snai1	5'GCAGCTGGCCAGGCTCTCGGTGGC 3	5'GTAGCTGGGTCAGCGAGGGCCTCC 3	65	35
r-Snai2	5' GACATCAGCAGTTCCATTCCAC 3'	5' CAAGGCAACGTGTGGGTCCG 3'	63	35
m,r-Sparc	5' ACACCCCCATGTGTGTGT 3'	5' AGCTTGTGGCCCTTCTTGGT 3'	60	30
m-Tgfa	5'TGGTGCAGGAAGAGAAGC 3'	5'TGACAGCAGTGGATCAGC 3'	59	30
m-Vim	5' TCCGCCAGCAGTATGAAAG 3'	5' TGGGTGTCAACCAGAGGAAG 3'	65	30
r-Vim	5' GGCCACCTCGTCCTTCG 3'	5' CACCCGGGGACGAGGAATAGA 3'	55	30
m,r-18S	5'GCGAAAGCATTTGCCAAGAA 3'	5'CATCACAGACCTGTTATTGC 3'	53	25

4.3. Quantitative Real Time PCR

RNA was obtained as explained in section 4.1. PCR reactions were done in duplicate in a 96-well plate, in a final volume of 25µL. Two methods were used: 1.-The TaqMan protocol and 2.-The Sybr Green protocol. In method 1.-, the reaction was prepared using 25µL of Taqman® Universal Master Mix, 2.5µL of predesigned TaqMan® primers (Table m.V), and 2µg of cDNA plus RNAse free water up to 50µL per duplicate. Gene expression was determined in an ABIPrism 7700 System following Manufacturer's protocol at the IDIBELL Serveis Tècnics Centrals. The levels of mRNA for each gene were normalized with the housekeeping gene 18S.

Table m.V. Predesigned Taqman® primers

Primer	Catalogue number
m-Bcl2l11	Mm00437796_m1
m-Bmf	Mm00506773_m1
h-SNAI1	Hs00195591_m1
h-18S	Hs03003631_g1
m-18S	Hs03003631_g1

In method 2.-, gene expression was determined in duplicates in an ABIPrism7700 System, using the Sybr® Green PCR Master Mix (Applied Biosystems). It was performed using 40 cycles, at a hypridization temperature of 58 to 62^{o}C and 18S was used to normalize the mRNA levels. The fold-changes for each gene expression levels were calculated using 2-ddCt. At the Table m.VI, the primer sequences are specified.

Table m.VI. Primer sequences used in Sybr® Green Real Time PCR

Gene	Forward primer	Reverse primer
h-SNAI1	5' CGAAAGGCCTTCAACTGCAAAT 3'	5' ACTGGTACTTCTTGACATCTG 3'
m,r-Snai1	5' CCACACTGGTGAGAAGCCA 3'	5' TCTTCACATCCGAGTGGGTTTG 3'
m,r-Sparc	5' ACACCCCCATGTGTGTGT 3'	5' AGCTTGTGGCCCTTCTTGGT 3'
h,m,r-18s	5' CGAGACTCTGGCATGCTAA 3'	5' CGCCACTTGTCCCTCTAAG 3'

4.4. Knock-down assays by RNA Interference

Small interfering RNA (siRNA) gene expression knock-down studies were performed using the TransIT-siQuest kit (Mirus, Madison, USA) and corresponding protocol. Each RNAi duplex (50nM) was transfected with Mirus transfection reagent (at a 1:300 dilution in complete medium) into SK-Hep1 and FaO cells for following the manufacturer's guidelines. After transfection cells were allowed to recover for 16 hours in complete medium, and then cells were trypsinized and plated for further experiments. Transient transfections were carried out following the protocol indicated in scheme Figure m.1A or Figure m.1B adjusted for Hep3B and neonatal murine hepatocytes because its sensitivity to trypsinitzation post-transfection.

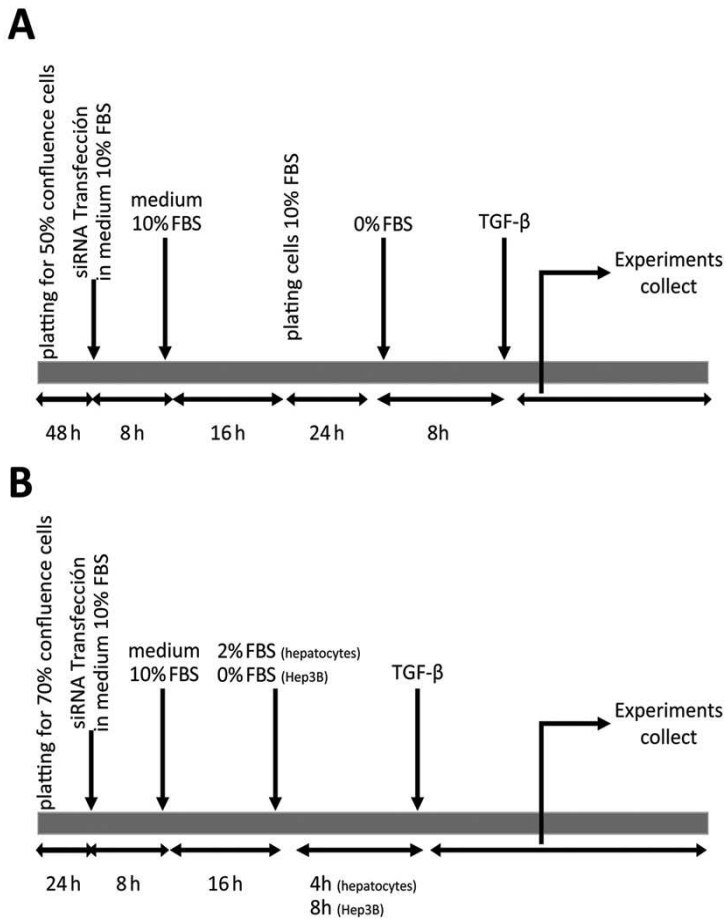


Figure m.1. SiRNA Transfection Protocol. A. SK-Hep1 and FaO cell lines. **B.** Hepatocytes and Hep3b cells.

Specific RNAi duplex were obtained from Sigma-Genosys (Suffolk, UK). The oligo sequences are shown in Table m.VII. The unspecific siRNA used was described in previous works (Sancho et al. 2006). Specific oligos with maximal knock-down efficiency were selected among three different sequences for each gene.

Table VII. siRNA sequences

Gene	sequence	Concentration
h-SNAI1	5' UCCCAGAUGAGCAUUGGCAGCGAGG 3'	50nM
m-Snai1	5'CAAACCCACUCGGAUGUGAAGAGAU 3'	50nM
r-Sparc	5' UGAAGAAGAUCCACGAGAA 3'	50nM
unespecific	5'GUAAGACACGACUUAUCGC 3'	50nM

5. Protein expression analysis

5.1. Obtention of total cell protein extract

Protein from cell extracts and conditioned medium was obtained from growing cells at experimental conditions. Then the media was collected and cells were scraped on ice with cold PBS. Media and cells were collected in a 15mL tube and then centrifuged at 2500 rpm for 5 min at 4^{o}C. The resulting pellet was washed with 2mL of PBS, and centrifuged at 2500 rpm during 5 minutes at 4°C. This pellet was resuspended in lysis buffer (Table m.VIII) and transferred to an eppendorf tube, the lysis was performed during 1 hour with rotation at 4°C. Then, the tubes were centrifuged at 13000 rpm during 10 minutes at 4°C, and the supernatants were collected and stored at -20°C until they were processed.

Table m.VIII. Lysis buffer

Sodium deoxicolate	1%
Tris-HCl pH7.4	10 mM
SDS	0.1 %
Triton-X-100	1%
NaCl	150 mM
EDTA	2 mM
PMSF	1 mM
Leupeptin	5 mg/mL
Na_3VO_4	0.1 mM
DTT	0.5 mM
β-glicerolphosphate	20 mM

5.2. Obtention of nuclear/cytoplasmatic proteins

Nuclear extracts were obtained from growing cells at experimental conditions. Cells were harvested and the pellet obtained from centrifugation at 1500 rpm for 5min at 4^{o}C was resuspended using 50 to 100µL of lysis Buffer A (Table m.IX) and then incubated on ice for 10 minutes with manual agitation every minute. After incubation, it was vortexed for 10 seconds and centrifuged at 10000 rpm for 2 min at 4^{o}C. The supernatant containing the cytosolic fraction was recovered and stored at -80°C until it's processed. The pellet was resuspended in 50 to 100µL of lysis Buffer B (Table m.X) and incubated at 4^{o}C for 20 minutes under smooth agitation. After incubation it was centrifuged at 13000 rpm for 3 min at 4^{o}C. The supernatant contains the nuclear fraction and it was stored at -80°C until it was processed.

Table m.IX. Lysis buffer A

Hepes-K pH7.9	10mM
KCl	10mM
$MgCl_2$	1.5mM
Dithiothreitol	0,5mM
PMSF	0.2mM
Leupeptin	2.5 µg/mL

Table m.X. Lysis buffer B

Hepes-K pH7.9	20mM
Glycerol	25%
NaCl	420mM
EDTA	0.2mM
$MgCl_2$	1.5mM
Dithiothreitol	0,5mM
PMSF	0.2mM
Leupeptin	2.5 µg/mL

5.3. Protein quantification by Bradford's method

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

5.4. 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 used the commercial kit BCA. For each measurement a standard curve of protein concentration was prepared with BSA in a range from 0 to 2µg/mL. The reaction was prepared by mixing Solution A and B in a ratio of 50:1 and 200µL of this mixture were added to 10µL of 1:10 diluted sample into a 96-well plate. After 30 minutes of incubation at 37°C, the absorbance was measured at the spectrophotometer at 595nm.

5.5. Protein immunodetection by Western Blot

Protein separation by their molecular weight was done by denaturalizing polyacrylamide gels. The protein samples were prepared by mixing 30 to 100 µg of protein with Laemmli buffer, and were denaturalized by heating them at 95°C. Once the samples were boiled, they were spinned and stored 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. Once the gel was ready it was assembled into the gel holder and immersed into the tank, which was filled with a electrophoresis buffer (25mM Tris-HCl; 0.1% SDS; 0.2M glycine; pH 8,3). Then the samples were carefully loaded to the gel together with a molecular weight standard. Then, they were submitted to electrophoresis at a constant voltage.

Once finished the electrophoresis, the proteins were transferred to a PVDF membrane through the passage of electrical current using a semi-dry equipment. The PVDF membrane was activated by its immersion into methanol for one minute following the manufacturer's instructions. Then the PDVF membrane and the Wattman paper were soaked in Transfer Buffer (Table m.XI) for 5 minutes, and the equipment was assembled as follows from bottom to top: 3 Wattman papers-PVDF membrane-Acrylamide gel-3 Wattman papers. An electrical current of 0.3mA was used during 0.5-1 hour. After this time, the membrane was 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 was washed several times in TTBS (Table m.XII).

Table m.XI. Transfer Buffer

Tris-HCl pH 8.3	48 nM
Glycine	39 nM
SDS	0.04 %
Methanol	20%

Table m.XII. TTBS

Tris-HCl pH 7.5	10 nM
NaCl	100 nM
Tween-20	0.05 %

The membrane was then incubated in 5% non fat dry milk in TTBS for 1 hour at RT. After this time, it was incubated with the primary antibody in 0.5% milk-TTBS at the dilution indicated at Table m.XIII for 16 hours at 4°C. Then the membrane was 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. The membrane was washed several times in TTBS. To visualize the antibody hybridized specifically to the protein of study, the membrane was incubated with a chemiluminescent solution, ECL, from Amersham Biosciences.

The secondary antibodies used were obtained from GE-Healthcare: Anti-Mouse (NA931V), anti-Rabbit (NA934V) and anti-Goat (P0449), which were conjugated with peroxidase.

Table XIII. Antibodies used for Western Blot

Primary antibody	Dilution	Secondary antibody	Purchased from
Phospho-Akt	1:1000	Rabbit	Cell Signaling 9271
β-Actin	1:3000	Mouse	Sigma AC-14 A5441
Bcl-xL	1:1000	Rabbit	Santa Cruz 634
Phospho-Erk1/2	1:1000	Rabbit	Cell Signaling 9101
Erk1/2	1:1000	Rabbit	Cell Signaling 9102
Mcl1	1:1000	Rabbit	Santa Cruz 819
Phospho-Smad2	1:1000	Rabbit	Cell Signaling 3101
Phospho-Smad3	1:1000	Rabbit	Cell Signaling 9520
Smad2/3	1:1000	Goat	Santa Cruz 6033
SPARC	1:1000	Mouse	AON-5031
Phospho-Src family	1:1000	Rabbit	Cell Signaling 2101

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

Table XIV. Stripping buffer

Tris HCl pH 6.8	62.5 nM
SDS	0.05 %
β-mercaptoethanol	100 nM

6. Immunostaining

6.1. Immunocytochemistry

Cells were plated on 2% gelatin-coated glass coverslips. For F-actin staining, after treatment, cells were 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, Cytokeratins and Vimentin cells were fixed with cold methanol for 2 min, blocked with 1% BSA and 10% fetal bovine serum in PBS for 1 hour and then incubated with the primary antibodies (Table m.XV). Primary antibodies were diluted 1:50 in 1% BSA and incubated for 2 hours at RT. For Bax, Bak and c-Kit staining, cells were fixed with 4% paraformaldehyde for 30 min at RT, permeabilized in PBS containing 0.1% triton X-100-0.1% BSA for 2min, blocked with 1%BSA and 10% fetal bovine serum in PBS for 1 hour and then incubated 1:50 (Bax, Bak) diluted in 1% BSA overnight at 4°C and 1:100 (kit) diluted in 1% BSA 2 hours at RT. After several washes with PBS, the samples were incubated with fluorescent-conjugated secondary antibodies (anti-mouse Alexa 488, anti-rabbit Alexa 488 or anti-rabbit Cy3-conjugated), at 1:200 for 1 hour at RT and embedded in Vectahield with

DAPI (Vector Laboratories, Burlingame, CA). Cells were visualized in an Olympus BX-60 microscope or a Leica DMR 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 edited in Adobe Photoshop.

Table m.XV. Antibodies used for Immunocytochemistry

Primary antibody	Purchased from
Albumin	Nordic Immunological
Bax	BD Pharmigen 6A7 clone
Bak	BD Pharmigen G317-2 clone
Cytokeratin 7	Dako M7018 OV-TL12130 clone
Cytokeratin 18	Progen 61028
Cytokeratin 19	Ab 1354
E-Cadherin	BD Transduction 610181
c-Kit	Santa Cruz 19619
Phalloidin	Sigma P1951
Vimentin	Sigma V6630

6.2. Hematoxylin-Eosin Staining

Mice tissues were fixed in 4% formalin from 4 to 16 hours, dehydrated (Ethanol 70% 1h at RT, Ethanol 96% 1h at RT (2 times), Ethanol 96% 16h at 4^{o}C, Ethanol 100% 1.5h at RT (3 times), Xilol 100% 1h at RT) and then embedded in paraffin 16 hours at 65^{o}C before mounting in the appropriate mold. Sections of 4µm-thick were unwaxed and hydrated (Xilol 100%, Ethanol 100%, Ethanol 96% 10 min at RT (3 times), Ethanol 70% 10 min at RT, distillated water 10 min at RT), stained with Hematoxylin (Sigma MHS32) from 15 seconds to 2 minutes and the staining was stopped and washed with tap water. After that, Eosin Staining was performed (Sigma HT110232) from 1 to 6 minutes and the staining was stopped and washed with tap water. Sections were dehydrated (Ethanol 70^{o} for 5 min at RT, Ethanol 96^{o} for 5 min at RT (3 times), Ethanol 100^{o} for 5 min at RT (3 times), Xilol 100^{o} for 10 min at RT) and mounted in DPX (BDH UN1307). Slices were visualized in an Olympus BX-60 microscope or a Leica DMR microscope and representative images were acquired with a Spot 4.3 digital camera and edited in Adobe Photoshop.

6.3. Tissue samples and Immunohistochemistry

Tissue samples from necropsied mice were formalin-fixed, dehydrated and then embedded in paraffin. For immunohistochemistry analysis, firstly sections of 4µm-thick were unwaxed and hydrated as section 6.2. Antigen retrieval was performed by boiling samples in Na-Citrate buffer (10mM, pH6) for 7 minutes in a pressure cooker and leaving it cool down to RT during 20 minutes into the citrate solution. Sections were incubated for 10 minutes in H_20_2 at 3%(v/v) to deactivate endogenous peroxides, then washed with distillated water and permeabilized for 10 min using a 0.1% Triton X-100-PBS solution. Slices were blocked for two hours (20% Fetal Bovine serum-BSA 2%) at RT and then incubated 16h with primary antibodies (diluted 1:50 or 1:100 in blocking solution) at 4^oC (Table m.XVI). After washing the unbound antibody with 0.1% Triton X-100-PBS, the sections were incubated with the corresponding, biotinylated secondary antibody. Antibodies were visualized using the Vectastain ABC kit with diaminobenzidine as substrate (Vector Laboratories, Burlingame, CA). Slices were counterstained with Hematoxylin and mounted (see section 6.2.).

Table XVI. Antibodies used for Immunohistochemistry

Primary antibody	Dilution	Purchased from
Cytokeratin 7	1:50	Dako M7018 OV-TL12130
Cytokeratin 18	1:100	Progen 61028
Cytokeratin 19	1:50	Ab 1354
E-Cadherin	1:100	BD Transduction 610181
KI67	1:100	Abcam 16667
P-Smad 2	1:100	Cell Signaling 3101
SPARC	1:100	Abcam 14071
Vimentin	1:100	Sigma V6630

6.4. TUNEL assay

Mice tissues were fixed, dehydrated and then embedded in paraffin as section 6.2. Sections of 4µm-thick were unwaxed and stained with DeadEndTM Colorimetric TUNEL System (Promega) following manufacturer instructions. Slices were visualized in an Olympus BX-60 microscope or a Leica DMR microscope and representative images were taken with a Spot 4.3 digital camera and edited in Adobe Photoshop.

7. Tumorigenicity and experimental metastasis assay in nude mice (xenography)

Athymic Balb/c nude mice were obtained from the Animal Production Area of Charles Rives (Lyon, France). Mice were maintained in pathogen free conditions (SPF) and used when they were 5 weeks old and weighted around 20g.

All procedures in Animal Experimentation described here have been approved and registered by DARP (Departament d'Agricultura Ramadería i Pesca, Generalitat de Catalunya) (Aproved procedure 2279 AFF) and all of them were performed at the Animal Facility of the IDIBELL (Nº B-990010) following the Institutional guidelines.

7.1. Subcutaneous cell injection

Tumor cells were harvested from subconfluent cultures (50-70% confluence) by trypsinization. The cells were washed in supplemented medium and then resuspended in Hanks' Balanced Salt Solution (HBSS) for injection. Only single-cell suspensions of greater than 90% viability (determined by Trypan blue dye exclusion) were used for the studies *in vivo*. Suspensions of cells were prepared at a concentration of $2x10^6$ viable cell/mL. Single inoculations of $1x10^6$ cells in 50µL of HBSS were used for subcutaneous inoculation into the flank using a 28-G needle.

Animals were examined for development palpable tumors and once detected, they were measured daily by using a vernier calliper. Tumor volume was calculated using the formula $V = (LxW^2) \times 0,5$ (being L the length and W the width). Tumors were excised when reaching 0.4 cm^3 volume by surgery (all surgeries were done in anaesthetized mice following the institutional guidelines) and the animals were kept alive until the experimental end-point and necropsed to investigate the presence of spontaneous metastasis. Tumors were processed for Hematoxylin and Eosin staining for histopathology examination. Both tumor growth and doubling time of the volumes were compared between groups.

7.2. Intrasplenic cell injection and experimental metastasis

Tumor cells were prepared as section 7.1. Single inoculations at $1x10^6$ cells/ 50µL in HBSS were used for intrasplenic inoculation into the spleen of anesthetized mice using a 28-G needle. After 48 hours, spleens were removed by surgery as described and performing a small incision on the abdomen to provide access to the spleen. The spleen is pulled out through the incision and curved forceps are used to hold it. Any pancreatic tissue was freed from the spleen by blunt dissection and was returned to the abdominal cavity. Once the splenic blood vessels were perfectly visible, we removed

the spleen by transecting them and prevented the bleeding vessels just pressing with a sterilized cotton swab before and after the removal. The wound was cleaned and the skin incision was closed using wound clips. All mice remained alive after surgery.

To analyze the metastatic development mice were killed at the experimental end-point: when became moribund or symptoms of tumors were detected (loose of weight, abdominal enlargement or presence of a peritoneal palpable mass) and all visceral organs were examined for the presence of tumor growth by gross and histological criteria. Livers were removed, screened for metastases and included in OCT and paraffin.

7.3. Obtention of tumor derived primary cultures

Single liver tumour foci from xenografts (see the procedures below) were excised from the total liver mass using a sterilized lancet and mechanically disaggregated. After that, we successively passed through 23-gauge needles to yield a single cell suspension. Cells were plated into complete medium at 20% fetal bovine serum on 12-well plates and were successively washed and trypsinized in order to clean the culture from fibroblasts or bloody cells. Primary cultures were maintained in the above described conditions and used at passage 3-5 after the establishment.

VII. RESULTS

1. Regulation by TGF-β of EMT-related genes that could control apoptosis in hepatocytes: Role of Snail and SPARC in TGF-β suppression function.

1.1. TGF-β induces an EMT process in immortalized neonatal murine hepatocytes (MH).

Taking into account the results obtained by our group in the recent years, we decided to explore TGF-β effects on immortalized neonatal murine hepatocytes (MH). These cells were obtained previously by our group (Gonzalez-Rodriguez et al. 2008) and they are a valuable tool for studying this cytokine effects on apoptosis and survival. These cells respond to TGF-β in a similar way than rat fetal hepatocytes do in terms of apoptosis and EMT induction (Valdes et al. 2002) and because they are an immortalized cell line, have more efficiency in transfection procedures and greatest reproducibility than a primary culture.

Previous results in our group indicate that in culture, fetal rat hepatocytes and FaO rat hepatoma cells, but not adult hepatocytes, suffer EMT in response to TGF-β (Valdes et al. 2002, Caja et al. 2007). Incubation of MH with TGF-β also induced apoptosis in these cells and the subpopulation that survived presented a fibroblastic-like phenotype clearly different from initial cultured hepatocytes.

In order to study the effects of TGF-β-induced EMT we chronically treated the MH with this cytokine, in the same way our group did in fetal rat hepatocytes (Valdes et al. 2002) and in FaO rat hepatoma cells (Bertran et al. 2009) to examine this phenomenon.

In collaboration with Esther Bertran PhD, MH were submitted to two cycles of TGF-β treatment (2ng/mL) in DMEM medium and in absence of FBS to isolate the resulting mesenchymal population, as indicated in Figure 1. After removing dead cells, the remaining ones were cultured in the same medium supplemented with 10% FBS (TβT-MH from TGF-β-treated immortalized murine hepatocytes). Cells that survived to TGF-β-induced apoptotic effect showed a mesenchymal phenotype, which was maintained when 0.5ng/mL of TGF-β was added to the culture medium.

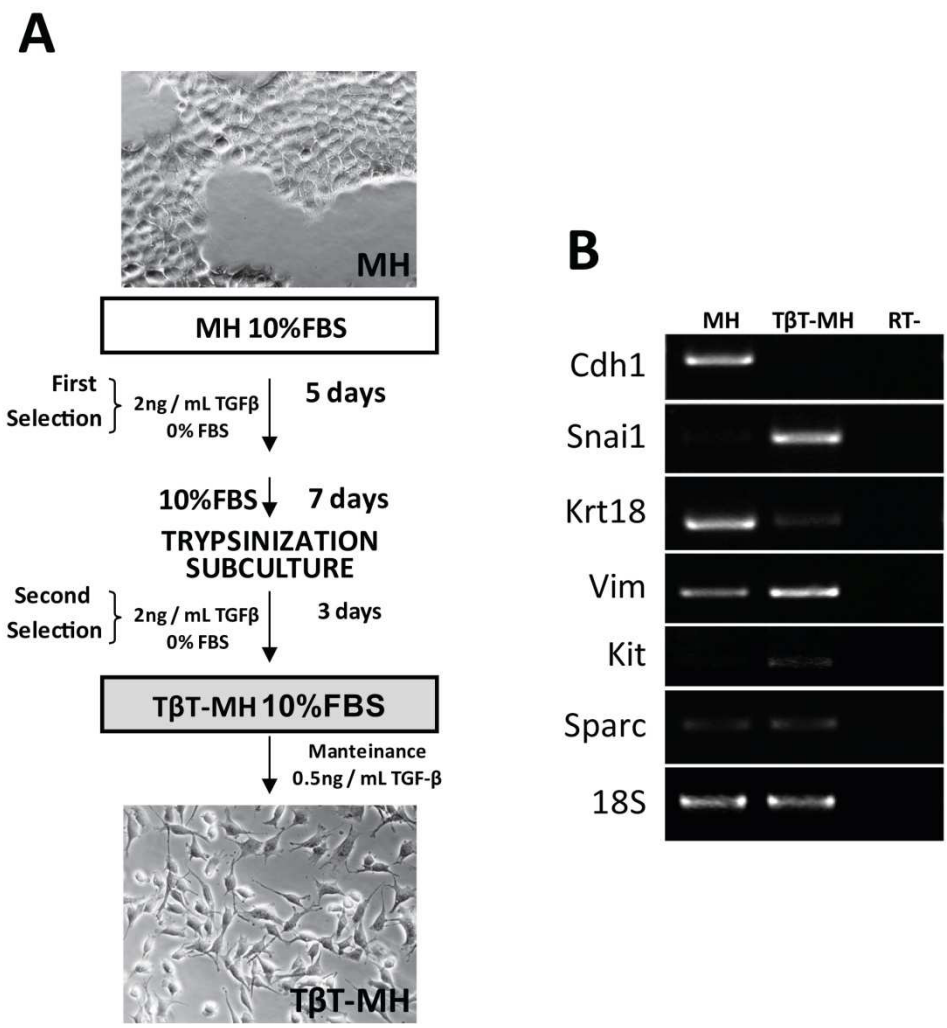


Figure 1. Characterization of immortalized neonatal murine hepatocytes (MH) that have undergone EMT in response to TGF-β. A. Isolation of immortalized murine hepatocytes that have survived to two cycles of TGF-β treatment, as indicated in the Figure (TβT-MH from TGF-β-treated immortalized murine hepatocytes). Phenotype is maintained when a low dose of TGF-β (0.5ng/mL) is added to the culture medium. Phase contrast photographs display the different morphologies of WT and TβT-WT hepatocytes. **B.** Transcript levels of *Cdh1* (E-cadherin), *Snai1* (snail), *Krt18* (Cytokeratin 18), *Vim* (Vimentin), *Kit* (c-kit) and *Sparc* were studied in MH and TβT-MH cells by semiquantitative PCR in order to determine changes related to the EMT process. *18S* levels were used as normalization control. Data are representative of three experiments.

Immortalized neonatal murine hepatocytes chronically treated with TGF-β (named TβT-MH) undergone EMT. We analyzed this phenomenon through semiquantitative PCR (Figure 1B) and immunofluorescence (Figure 2). As indicated in (Figure 2A), there is an F-actin cytoskeleton reorganization, from a pericellular localization in MH cells to a redistribution and apparition of stress fibers in TβT-MH cells. In correlation with an EMT process, E-cadherin (*Cdh1*) expression is lost concomitant with an upregulation of Snail (*Snai1*) in TβT-MH cells compared to MH cells (Figures 1B, 2B). There was a

replacement of the intermediate filaments from Cytokeratin 18 (*Krt18*) to Vimentin (*Vim*) (Figures 1B, 2C), but we didn't observe any changes in *Sparc* levels (Figure 1B). Interestingly TβT-MH cells also induced the expression of c-kit, the stem cell factor receptor, in paralell to the EMT process (Figure 1B).

When neonatal murine hepatocytes were isolated and immortalized, a subsequent characterization was performed to assure the resulting cells were indeed hepatocytes with Albumin (*Alb*) detection (Valverde et al. 2003). This plasma protein is secreted exclusively by hepatocytes and both MH and TβT-MH hepatocytes expressed albumin as it's shown in Figure 2D, so they are still hepatocytes.

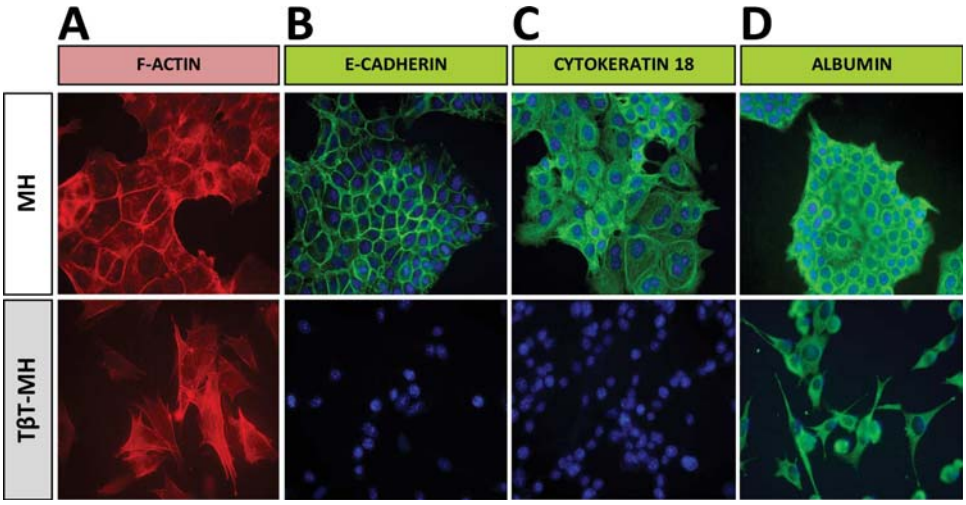


Figure 2. Chronic TGF-β treatment induces an EMT process in immortalized murine hepatocytes. A. F-actin filaments were stained with rhodamine-conjugated phalloidin in MH and TβT-MH hepatocytes. **B-D.** Immunofluorescence detection of E-cadherin, Cytokeratin 18 and albumin respectively in both cell types. Blue signal represents the nuclear DNA-staining with DAPI. Photographs at 60X magnification are representative of three experiments.

Previous results of our group in fetal rat hepatocytes (Valdes et al. 2002) and in FaO rat hepatoma cells (Caja et al. 2007) indicated that the EMT process induces survival to the cells, promoting the resistance to the TGF-β-induced cell death. Moreover, previous data of our group also indicated that Snail blocks proliferation and confers resistance to cell death (Vega et al. 2004).

These results led us to study which might be the molecular mechanisms involved in TGF-β-induced EMT, which might confer resistance to its pro-apoptotic effects, focusing first in the role of Snail. To that purpose, we targeted knock-down *Snai1* levels using a specific siRNA as described in the Materials and Methods.

1.2. Effects of *Snai1* knock-down in TGF-β-induced EMT in immortalized neonatal murine hepatocytes.

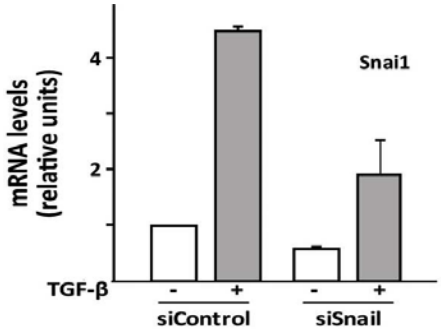


Figure 3. Snai1 downregulation attenuates its induction by TGF-β. Immortalized neonatal murine hepatocytes were transfected with either an unspecific siRNA (siControl) or the specific murine *Snai1* siRNA, then were treated with TGF-β (2ng/mL) for 6 hours and analyzed by Real Time PCR. Data are mean ±SEM of three experiments.

Hepatocytes were easily transfected with efficiencies around 80%. Results indicated that *Snai1* upregulation induced by TGF-β was significantly reduced when cells were transfected with specific siRNA for *Snai1* in comparison with those cells transfected with a control siRNA (Figure 3).

Under these circumstances, downregulation of the expression of epithelial markers like E-cadherin induced by TGF-β was impaired after Snai1 knock-down (Figure 4A). Changes in the distribution of F-actin cytoskeleton were also analyzed, finding that stress fibers produced by TGF-β were prevented when hepatocytes were transfected with siSnail (Figure 4B). To assess MH still were hepatocytes we tested Albumin expression (Figure 4C), finding no changes in its levels. Results indicated that TGF-β-induced EMT is impaired in immortalized neonatal murine hepatocytes after *Snai1* cancelation.

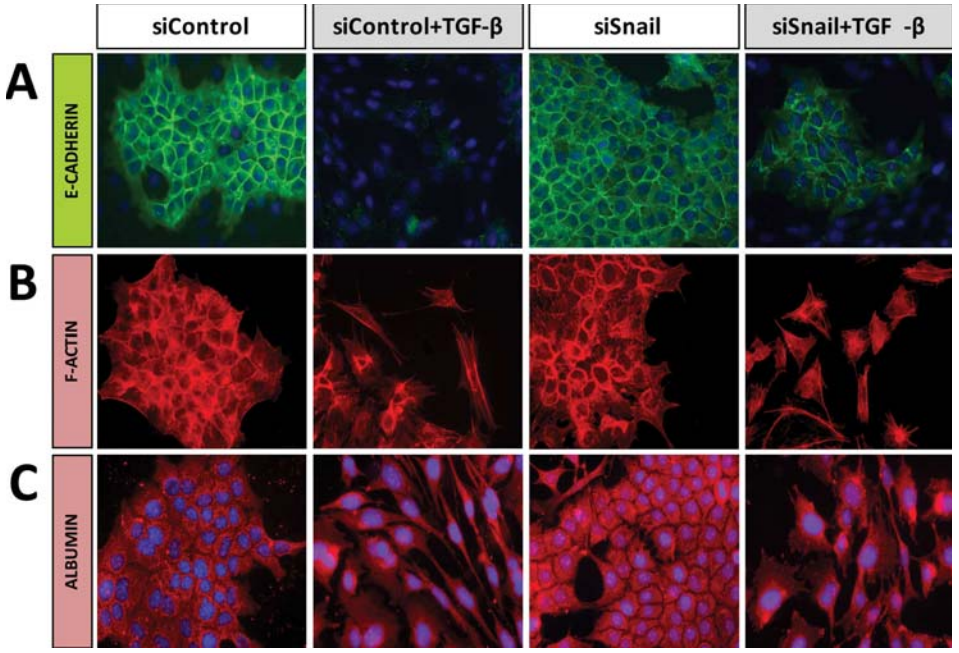


Figure 4. Snai1 cancelation impairs TGF-β-induced-EMT in neonatal murine hepatocytes. Hepatocytes were treated as described in Figure 3. **A,C.** Immunofluorescent detection of E-cadherin and Albumin after 30 hours of TGF-β treatment. Blue signal represents the nuclear DNA staining with DAPI. B. F-actin filaments were stained with rhodamine-conjugated phalloidin. Representative images from three independent experiments at 60X magnification are shown.

1.3. Effects of Snai1 downregulation in the TGF-β-induced apoptosis in immortalized neonatal murine hepatocytes.

In order to explore the contribution of Snai1 into the pro-apoptotic effects mediated by TGF-β in immortalized murine hepatocytes, we cancelled *Snai1* through targeting knock-down with specific siRNA (Figure 3).

TGF-β-treated control cells depicted a clear apoptotic process (Figure 5B,C) that produced cell death at later times (Figures 5A,E). Upon Snail downregulation, treatment with TGF-β induced a higher level of Caspase 3 activity and apoptotic nuclei (Figures 5B,C) that gave rise to a massive cell death after 48 hours (Figures 5A, E). Interestingly, at this time, the only hepatocytes that survived were those showing a mesenchymal phenotype, indicative of those that, albeit probably reduced, still had enough levels of Snail and had finally undergone EMT (Figures 5A,E).

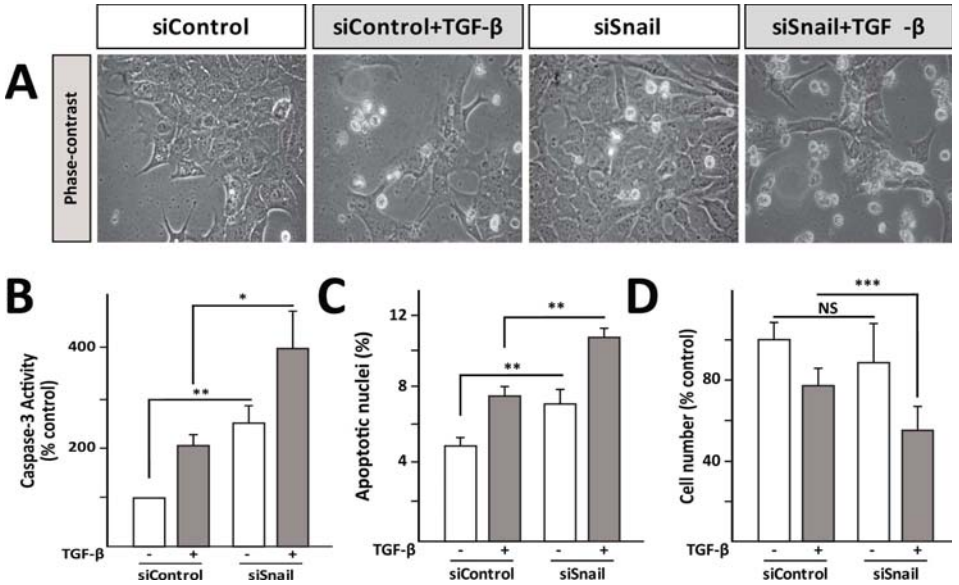


Figure 5. Snai1 downregulation enhances the apoptosis response to TGF-β in immortalized murine hepatocytes. Hepatocytes were treated as described in Figure 3. **A.** Phase-contrast photographs of similar cultures after 48 hours of TGF-β treatment. **B.** Caspase 3 activity was analyzed after 30 hours of TGF-β treatment by fluorimetry and presented as % versus siControl. **C.** Apoptotic nuclei 30 hours after TGF-β treatment (percentage of condensed and/or fragmented nuclei assessed by Hoechst staining). **C.** Cell viability after 48 hours treatment analyzed by Crystal Violet staining and expressed as a % versus siControl. Data are mean ±SEM of three experiments. In **C.** 10-15 different fields per condition were counted in each experiment (n=3). Student's T-test: *P<0.05; **P<0.01; ***P<0,001; NS: no significant.

Experiments in collaboration with Patricia Sancho PhD in our lab were performed to confirm that the massive cell death observed in the presence of Snail1 siRNA was specifically due to *Snai1* downregulation. A plasmid containing the coding region of *Snai1* (Snail1CD) was used and a siRNA specific for the 3' untranslated sequence of *Snai1* (Snail1-3') was designed so that it could not target Snail1CD (results not shown). This construct was able to prevent the cell death induced by TGF-β in control cells and when si*Snail3*' was expressed together with Snail1CD, it very significantly attenuated the action of the siRNA.

Next we wanted to know which could be the intracellular signals involved in the Snail-mediated survival in immortalized neonatal murine hepatocytes.

In our model of neonatal murine hepatocytes, TGF-β induces the expression of EGFR ligands, such as transforming growth factor-alpha (*TGF-α*) and heparin-binding epidermal growth factor-like (*HB-EGF*) (Figure 6A) and Snai1 downregulation impairs this effect. This result correlates with the inhibition of Akt, Erks and c-Src family phosphorylation promoted by TGF-β (Figure 6B) after Snai1 silencing.

The blockage of typical survival pathways after Snai1 downregulation indicates that Snai1 plays an important role in the survival signals mediated by TGF-β in hepatocytes.

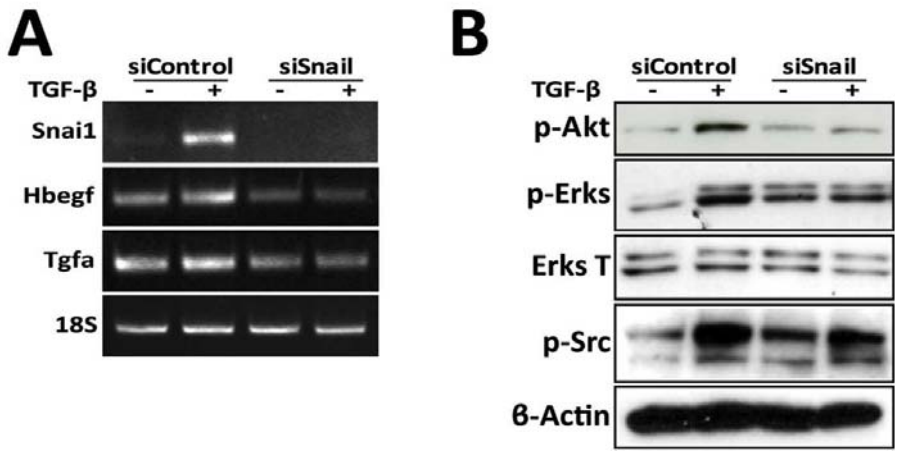


Figure 6. Snai1 downregulation blocks the TGF-β-induced EGFR ligands expression and inhibits the phosphorylation of Akt, Erks and c-Src family. Hepatocytes were treated as described in Figure 3.**A.** Levels of *Tgfa* and *Hbegf* transcripts analyzed by semi-quantitative PCR after 6h of TGF-β treatment. *Snai1* levels are shown as a control of downregulation and *18S* levels are shown as a loading control. **B.** Western Blot analysis of total Erks and phosphorylated levels of Akt, Erks and c-Src family after 12 hours of TGF-β treatment. β-Actin levels were used as loading control. A representative experiment of three is shown.

Next we decided to investigate whether Smad signaling could be affected by *Snai1* interference. Silencing *Snai1* in neonatal murine hepatocytes induced Smad3 phosphorylation at 30 minutes of TGF-β treatment while there were still no response in hepatocytes transfected with siControl (Figure 7).

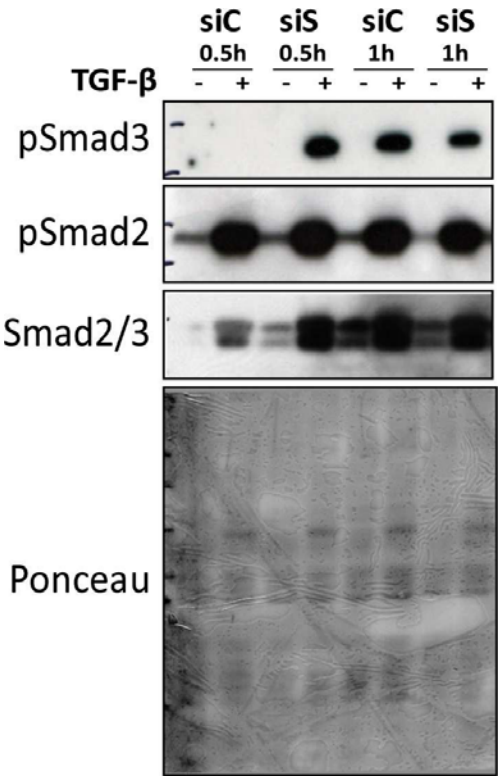


Figure 7. Snai1 silencing induces an earlier Smad3 phosphorylation on cells treated with TGF-β. Cells were treated as described in Figure 3. Phosphorylation and total levels of Smad 2 (upper band) and Smad3 (lower band) were analyzed in the presence or absence of TGF-β for the times indicated. Ponceau staining was used as a loading control. A representative experiment of three is shown.

Smad2 phosphorylation by TGF-β was similar regardless Snail expression was knock-down. Antiapoptotic signals induced by TGF-β correlated with an increase in the intracellular content of anti-apoptotic proteins of the Bcl-2 family, Bcl-xL and Mcl1. A similar response was observed in this murine neonatal liver cell line (Figure 8A), which was attenuated when *Snai1* levels were decreased with siSnail (Figure 8A). Effects were better observed at the protein level, which suggested that both transcriptional and post-transcriptional mechanisms are involved.

In the presence of siRNA for *Snai1*, in addition to an increase in cell death, we observed an earlier and stronger upregulation of genes previously related to the apoptosis induced by TGF-β, such as the BH3-only genes Bim (*Bcl2l11*) and *Bmf* (Ramjaun et al. 2007) (Figure 8B, C). Interestingly, *Snai1* interference is sufficient to upregulate *Bim* expression, a BH3-only protein that responds to the absence of survival signals, whereas the expression of *Bmf* is significantly upregulated in response to TGF-β when Snail is downregulated. Coincident with these changes in gene expression, the percentage of cells exposed to TGF-β with activated Bax or Bak significantly increased in the presence of Snai1 siRNA, indicating that *Snai1* can counteract the mitochondrial-dependent apoptosis induced by TGF-β in hepatocytes (Figure 8D).

Indeed, *Snai1* downregulation provokes changes in the TGF-β-mediated regulation of Bcl-2 family, favoring the increase in the ratio of the pro-apoptotic versus anti-apoptotic members. All these results indicate that *Snai1* can counteract the mitochondrial-dependent apoptosis induced by TGF-β in hepatocytes.

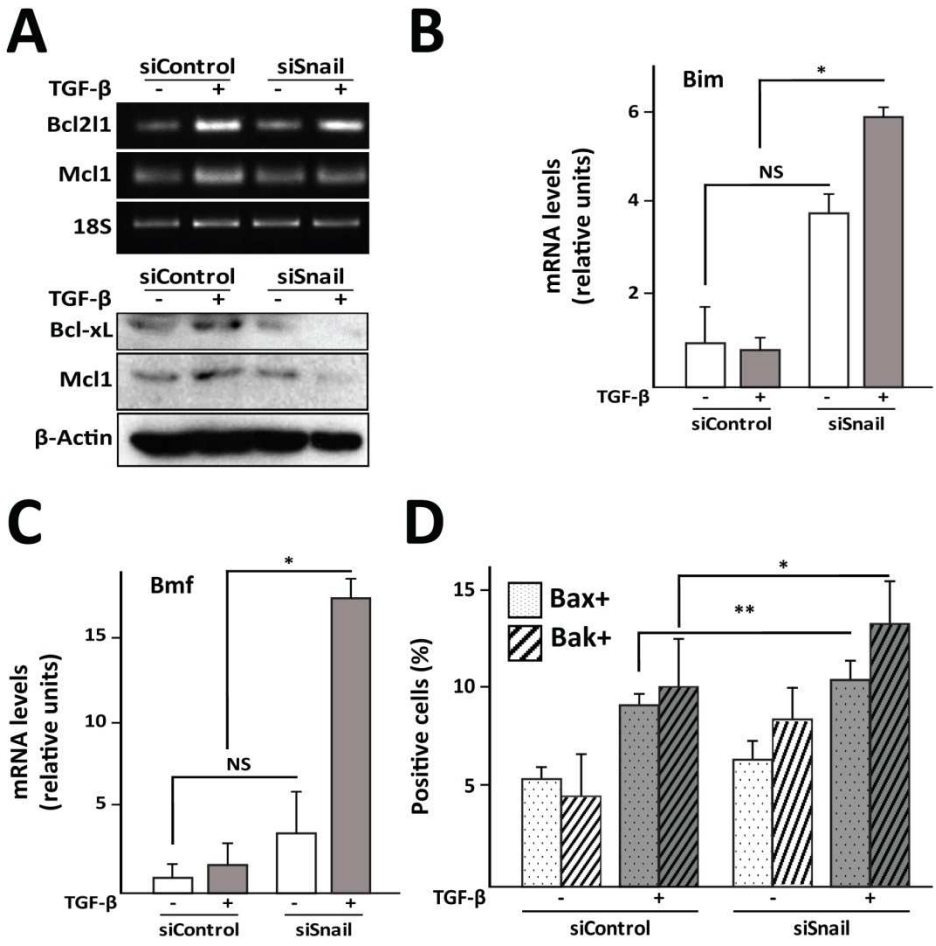


Figure 8. Snai1 downregulation increases the mitochondrial-dependent apoptotic events induced by TGF-β in hepatocytes. Cells were treated as described in Figure 3.**A.** Levels of Bcl-xL (*Bcl2l1*) and *Mcl1* transcripts analyzed by semi-quantitative PCR after 6h of TGF-β treatment. *18S* transcripts are shown as control. Mcl1 and Bcl-xL protein levels after 24 hours of TGF-β treatment. β-Actin levels are shown as a loading control. A representative experiment of three is shown. **B, C.** Analysis of the levels of pro-apoptotic *Bim* and *Bmf* transcripts by Real Time PCR after 3 hours of TGF-β treatment. Data correspond to five independent experiments. **D.** Percentage of cells presenting the active form of BAX or BAK after 6 hours of TGF-β treatment and analyzed by immunofluorescence as described in the Materials and Methods. Data are means ±S.E.M. of a representative experiment (n=3), where 25 independent fields per condition were counted. Student's T-test: **P*<0.05; ***P*<0.01; NS: no significant.

Having confirmed that *Snai1* plays an important role in the resistance to apoptosis induced by TGF-β, we examined whether it might also protect hepatocytes from other death mechanisms. We chose to analyze death by anoikis (detachment-induced cell death) because of the relevance of anoikis resistance in metastatic processes.

The results indicated that a decrease in *Snai1* expression in immortalized murine hepatocytes impaired the TGF-β-induced cell adhesion in the absence of substrate. The number of adherent cells, reflecting their capacity to attach to untreated plastic, only increased on exposure of control hepatocytes to TGF-β but not in those with diminished Snai1 expression (Figure 9, left). Furthermore, when the cells that did not adhere were plated on cell culture dishes to analyze their capacity to survive for 48 hours in the absence of substrate, those cells transfected with specific *Snai1* siRNA were more susceptible to die than control cells, both in the presence or absence of TGF-β (Figure 9, right).

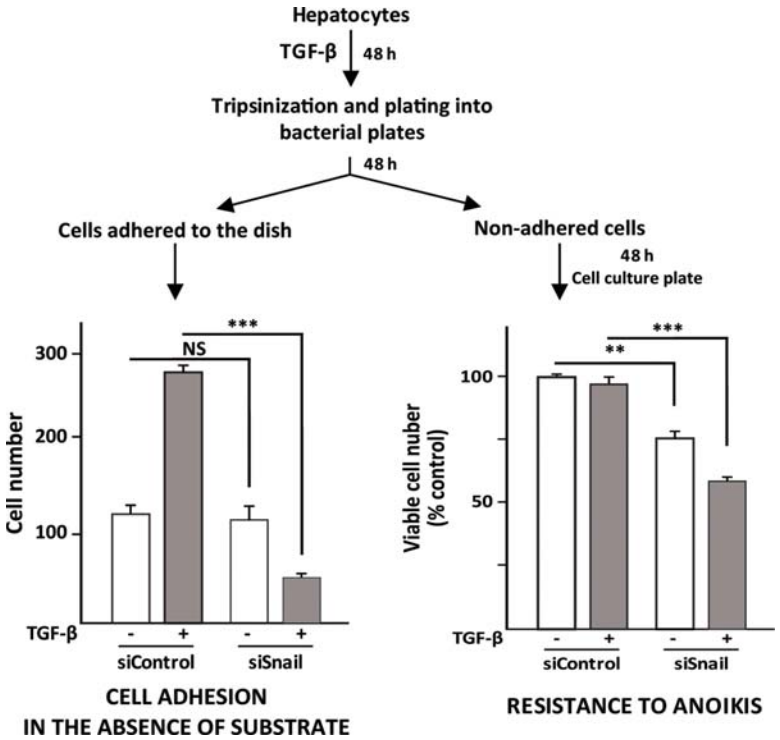


Figure 9. Snai1 downregulation sensitizes immortalized murine hepatocytes to cell death by anoikis. Cells were transfected with either a non-specific siRNA (siControl) or a *Snai1* siRNA (siSnail) as described in the Materials and Methods and were then treated with TGF-β (2 ng/mL) for 48 hours. Cells were subsequently trypsinized and plated on bacteria dishes for 48 hours. Left: Number of cells that adhered to the bacteria dishes (counted after Crystal Violet staining), reflecting their capacity to attach on non-treated plastic. Data are means ± S.E.M. of a representative experiment (*n*=3; 25 independent fields). Right: Cells that did not adhere to the bacteria dishes were later plated at identical cell density on cell culture dishes. Viable cells were quantified after 48 hours (Crystal Violet staining and spectrophotometric analysis). siSnail cells were sensitized towards apoptosis in the absence of cell adhesion (anoikis). Data are means ± S.E.M. of a representative experiment (n=3). Student's T-test: **P<0.01; ***P<0.001; NS: no significant.

1.4. Effects of *Snai1* downregulation in the TGF-β-induced apoptosis in human hepatocellular carcinoma cells: Relevance in human liver tumorigenesis.

Next, we wondered whether *Snail1* could be responsible for the resistance to TGF-β-induced apoptosis in human hepatocellular carcinoma cells, which might be relevant for the progression of human liver tumorigenesis (Fabregat 2009). We tested the response to *Snai1* downregulation of two different human hepatocellular carcinoma cell lines: Hep3B and SK-Hep1.

The Hep3B cell line, although shows a poor response to TGF-β in terms of apoptosis (Caja et al. 2009, Caja et al. 2011b), they are also susceptible to express *Snai1* and undergo EMT in a similar way to fetal hepatocytes upon TGF-β exposure (Caja et al. 2011a). Without serum, Hep3B cells show autocrine growth as they express survival signals that attenuate TGF-β-induced cell death (not shown results).

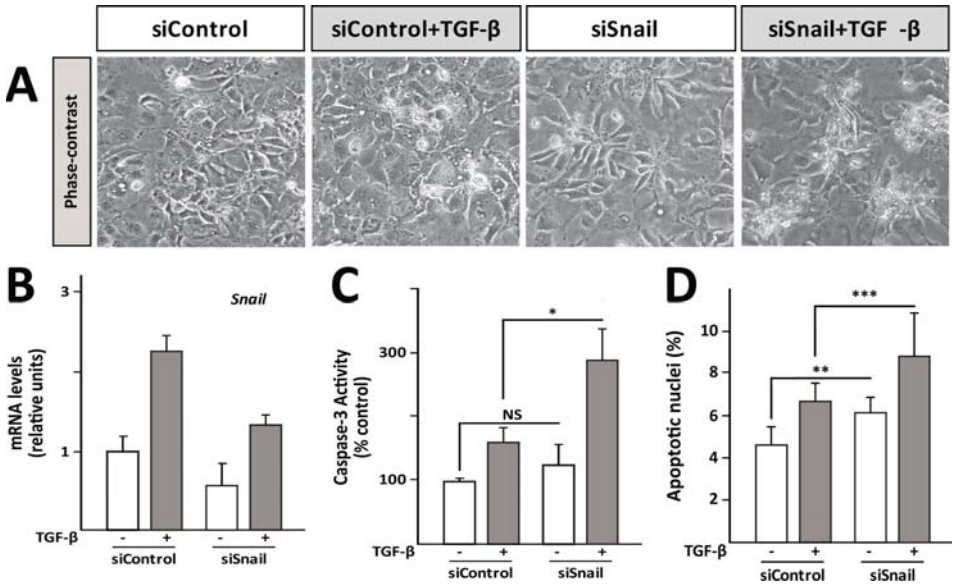


Figure 10. Snai1 enhances the apoptotic effects of TGF-β in the hepatocellular carcinoma cell line Hep3B. Hep3B cells were transfected with either an unspecific siRNA (siControl) or the specific human *Snai1* siRNA, as described in the Materials and Methods, and then were treated with TGF-β (2ng/mL). **A.** Photographs of hep3B cell culture after 36 hours of TGF-β treatment (one representative experiment of three). **B-D.** In all cases, cells were treated with TGF-β over 24 hours. **B.** *Snai1* transcript levels analyzed by Real Time PCR after a 1 hour treatment with TGF-β. **C.** Caspase 3 activity was analyzed by fluorimetry and presented as the percentage of the controls. **D.** Percentage of apoptotic nuclei (microscopy observation of condensed and/or fragmented nuclei after Hoechst staining; 15 independent fields/dish). Data are the means ± S.E.M. of at least three independent experiments and they were compared as indicated in the Figure. Student's T-test: *P<0.05; **P<0.01; ***P<0.001; NS: no significant.

When *Snai1* was downregulated in these cells, TGF-β was able to efficiently induce cell death (Figure 10A) assessed by the increased levels of Caspase 3 activation (Figure 10C) and the percentage of apoptotic nuclei (Figure 10D), which correlated with diminished levels of *Snai1* induction after TGF-β administration (Figure 10B).

The SK-HEP1 cell line is a liver adenocarcinoma cell line which shows an endothelial-like phenotype and a late TGF-β signature, according to Coulouarn and colleagues (Coulouarn et al. 2008). This cell line has the ability to up-regulate the expression of mesenchymal genes after TGF-β treatment (Caja et al. 2011b), has autocrine production of TGF-β and as it's shown in Figure 11A-D, resistance to TGF-β-induced cell death. In collaboration with Sonia Vega phD, we tested *Snai1* downregulation in these cells finding that its cancellation sensitize them to apoptotic features that are enhanced in the presence of additional extracellular TGF-β.

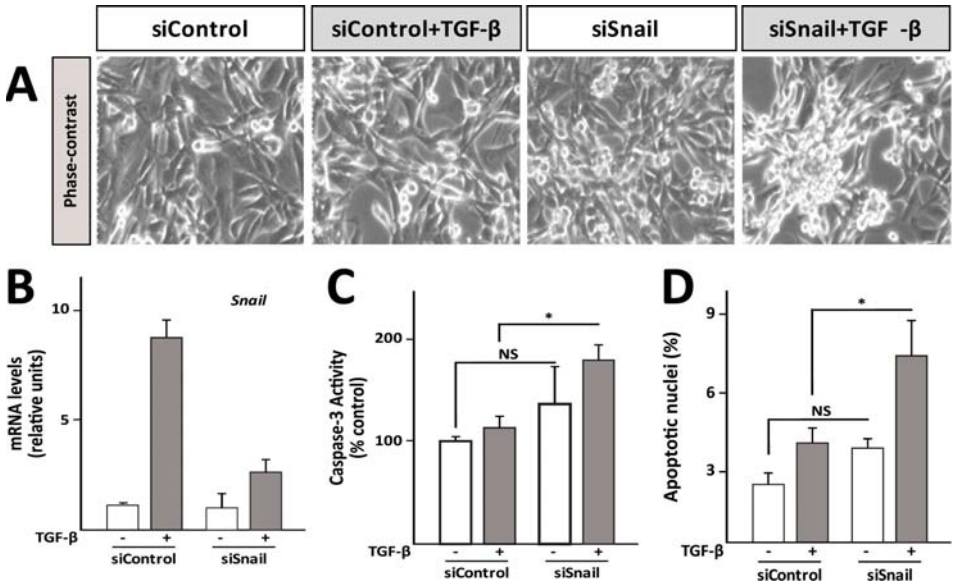


Figure 11. Snai1 downregulation restores the apoptotic response to TGF-β in human liver adenocarcinoma cell line SK-Hep1. SK-Hep1 cells were transfected as described in Figure 10. **A.** Photographs of SK-Hep1 cell culture after 36 hours of TGF-β treatment (one representative experiment of three). **B-D.** In all cases, cells were treated with TGF-β over 24 hours. **B.** *Snai1* transcript levels analyzed by Real Time PCR after a 1 hour treatment with TGF-β. **C.** Caspase 3 activity was analyzed by fluorimetry and presented as the percentage of the controls. **D.** Percentage of apoptotic nuclei (microscopy observation of condensed and/or fragmented nuclei after Hoechst staining; 15 independent fields/dish). Data are the means ± S.E.M. of at least three independent experiments and they were compared as indicated in the Figure. Student's T-test: *P<0.05; NS: no significant.

Altogether, these results indicate that malignant cells can circumvent the death-inducing effect of TGF-β by directing it towards the induction of *Snai1* expression, which promotes both EMT and survival.

1.5. Analysis of SPARC expression in immortalized neonatal murine hepatocytes and FaO rat hepatoma cell line.

Different reports have associated induction of SPARC to both EMT and survival signals (Robert et al. 2006, Shi et al. 2004). However, nothing was known about the role of SPARC in the regulation of TGF-β-induced EMT and apoptosis in hepatocytes. Immortalized neonatal murine hepatocytes treated with TGF-β don't up-regulate *SPARC* (Figure 1B). In contrast, a liver tumor cell line, such as FaO rat hepatoma cells, induced *SPARC* in response to TGF-β treatment. We chose the FaO rat hepatoma cell line because we previously showed that this cell line can induce apoptosis and also an EMT process in response to TGF-β (Caja et al. 2007). As it's shown in Figure 12B, FaO cells induced *SPARC* in response to TGF-β treatment (experiments performed in collaboration with Estrella Molla in our group).

Indeed, a tumoral liver cell line like the FaO rat hepatoma cells, but not non-tumoral liver cell line like the neonatal immortalized murine hepatocytes, up-regulated *SPARC* in response to TGF-β, which associates TGF-β-induced SPARC to liver tumorigenesis.

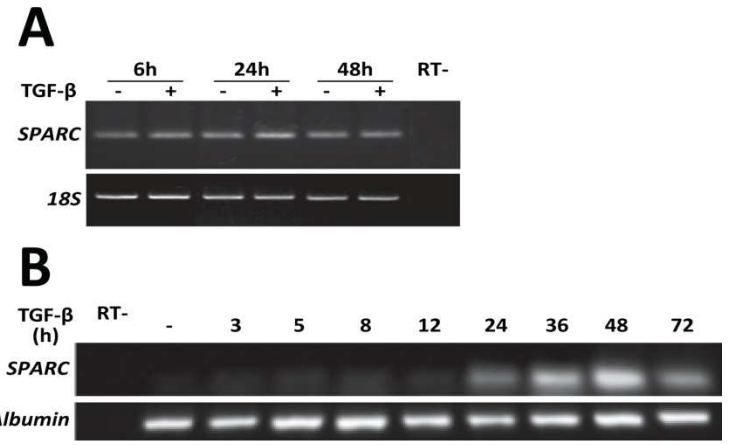


Figure 12. TGF-β induces SPARC expression in FaO cells but not in immortalized neonatal murine hepatocytes. A. Levels of *SPARC* transcripts in immortalized neonatal murine hepatocytes analyzed by semi-quantitative PCR after TGF-β treatment (2ng/mL) at the indicated times in the Figure. *18S* transcripts are shown as control. **B.** Levels of *SPARC* transcripts in FaO rat hepatoma cells analyzed by semi-quantitative PCR after TGF-β treatment (2ng/mL) at the indicated time in the Figure. *Albumin* transcripts are shown as control. A representative experiment of three is shown.

1.6. Effects of SPARC downregulation in the TGF-β-induced EMT in FaO rat hepatoma cells.

Since we wanted to study the effects of SPARC downregulation in the EMT process and FaO cells can induce SPARC and EMT in response TGF-β, we use this model to analyze this phenomenon.

FaO rat hepatoma cell were transfected with either an unspecific siRNA or the specific rat *SPARC* siRNA as described in the Materials and Methods. Results in Figure 13 indicated that SPARC induction by TGF-β was heavily reduced in the presence of the specific *SPARC* siRNA.

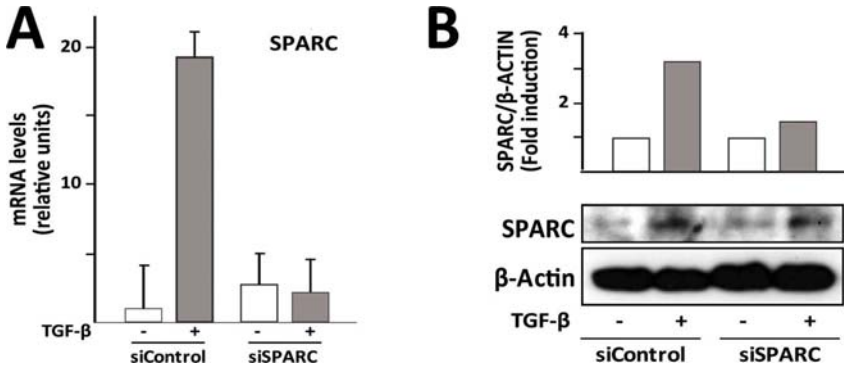


Figure 13. siRNA targeted knock-down of SPARC reduces TGF-β-mediated induction in FaO rat hepatoma cells. FaO rat hepatoma cells were transfected with either an unspecific siRNA (siControl) or the specific rat *SPARC* siRNA, as described in the Materials and Methods, and then were treated with TGF-β (2ng/mL). **A.** *SPARC* transcripts analyzed by Real Time PCR after 24 hours of TGF-β treatment. Data are means ±S.E.M. of 3 experiments. **B.** SPARC protein levels analyzed by Western blot (24 h of treatment). β-Actin was used as loading control. Densitometric analysis of a representative experiment (*n*=3) is shown in the graph and presented as the % versus control.

When FaO cells were incubated with TGF-β, the majority of surviving cells acquired a fibroblastic morphology. After *SPARC* knock-down, the downregulation and delocalization of E-Cadherin expression induced by TGF-β was clearly impaired and the EMT process was prevented after 30 hours of treatment, as it's shown in Figure 14A. The F-actin cytoskeleton reorganization and formation of stress fibers induced by TGF-β was prevented when FaO cells were transfected with siSPARC (Figure 14B). Results indicate that TGF-β-induced EMT is decreased in FaO rat hepatoma cells after attenuation of *SPARC* expression.

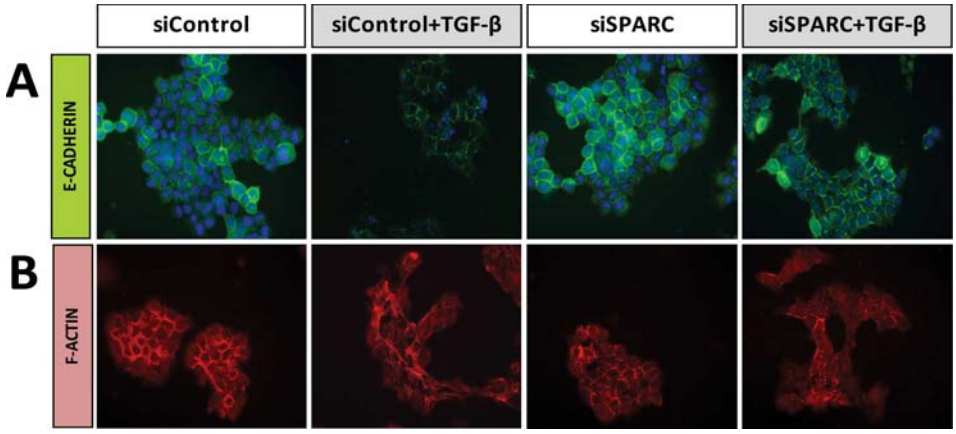


Figure 14. SPARC cancelation attenuates TGF-β-induced EMT in FaO rat hepatoma cells. FaO cells were treated as described in Figure 13. **A.** Immunofluorescent detection of E-cadherin after 30 hours of TGF-β treatment. Blue signal represents the nuclear DNA staining with DAPI. **B.** F-actin filaments were stained with rhodamine-conjugated phalloidin. Representative images from three independent experiments are shown.

1.7. Implication of SPARC in TGF-β-induced apoptosis in FaO rat hepatoma cells.

Having studied the role of *Snai1* in TGF-β-induced apoptosis in immortalized neonatal murine hepatocytes we wonder whether *SPARC* could also have a role in the apoptotic TGF-β signaling in FaO rat hepatoma cells. We studied the effect of cancelling *SPARC* in the suppressing functions of TGF-β in FaO cells.

TGF-β-treated control cells showed apoptotic features measured as activation of Caspase 3 Activity (Figure 15B) which preceded and increase in the percentage of hypodiplod cells (Figure 15C) that produce cell death at later times (Figure 15A). Upon *SPARC* cancelation, TGF-β-induced Caspase 3 Activity was significantly increased and also the percentage of cells with hypodiploid DNA content. In Figure 15D it can be observed that *SPARC* downregulation by itself induced cell cycle arrest in G0/G1 phase (shown as an increase in the percentage of cells in this phase of the cell cycle) as TGF-β does in FaO cells, in agreement with previous reports of our group (Caja et al. 2007).

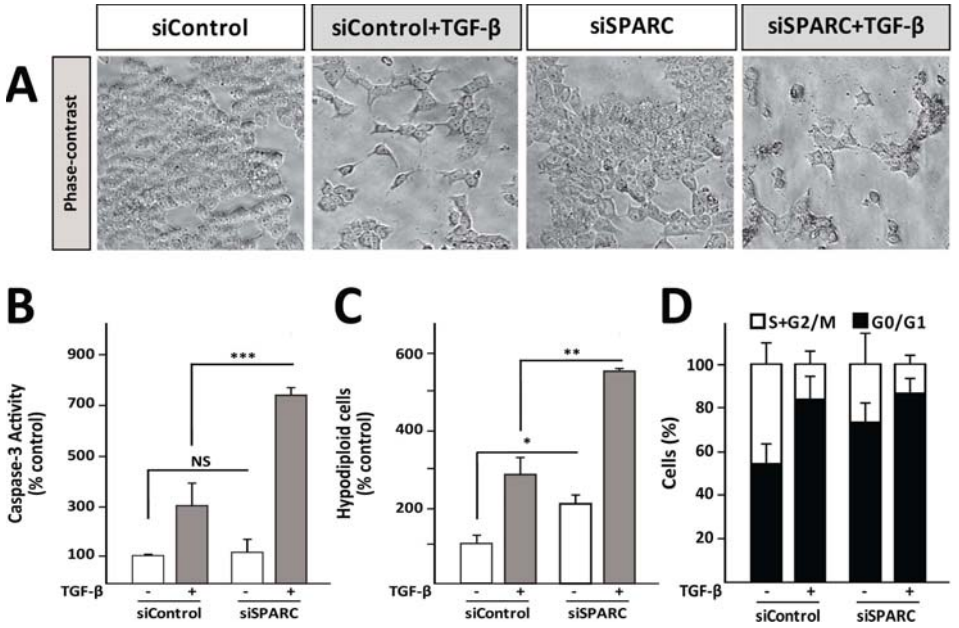


Figure 15. SPARC downregulation enhances TGF-β induced apoptosis and induces cell cycle arrest in FaO rat hepatoma cells. FaO cells were treated as described in Figure 13. **A.** Photographs of FaO cells in culture after 48 hours of TGF-β treatment (one representative experiment of three). **B.** Caspase 3 activity was analyzed after 24 hours of TGF-β treatment by fluorimetry and presented as the % versus control. **C.** DNA content, analysed by flow cytometry after 36 hours of TGF-β treatment. The percentage of hypodiploid (apoptotic) cells is shown. **D.** Percentage of cells in each phase of the cell cycle after 36 hours of TGF-β treatmet. Data are the means ± S.E.M. of three independent experiments and they were compared as indicated in the Figure. Student's *T*-test: **P*<0.05; ***P*<0.01; ****P*<0.001; NS: no significant**.**

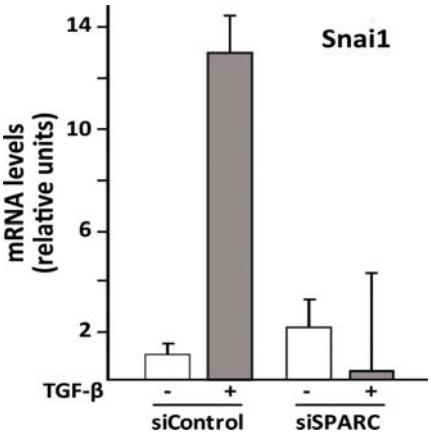


Figure 16. *SPARC* silencing impairs Snai1 upregulation by TGF-β in FaO cells. FaO cells were treated as described in Figure 13. *Snai1* transcripts analyzed by Real Time PCR after 24 hours of TGF-β treatment. Data are means ±S.E.M. of 3 experiments.

All these results led us to wonder whether there was an intimate relation between Snai1-TGF-β-SPARC.

To answer this question we targeted *Snai1* in FaO rat hepatoma cells, finding no changes in *SPARC* expression. Inversely, we tested *Snai1* expression in FaO cells where we canceled *SPARC*. As it's shown in Figure 16, we found that when we canceled *SPARC* expression, Snai1 upregulation triggered by TGF-β was impaired in these FaO rat hepatoma cells.

2. Tumorigenesis of *in vitro* TGF-β-chronically-treated tumor liver cells. Morphological and phenotypical analysis and apoptotic response.

2.1. Study of the metastatic potential of TGF-β-chronically-treated-FaO hepatoma cells by subcutaneous injection.

To isolate the mesenchymal population resulting from TGF-β-induced-EMT, FaO cells were submitted to two cycles of TGF-β treatment (2ng/mL), as indicated in the Figure 17 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. The addition of 0.5 ng/mL of TGF-β to the culture medium was enough to maintain the mesenchymal phenotype. However, the TβT-FaO cells cultured in the absence of TGF-β reverted to an epithelial phenotype in 3-4 weeks (Rev-FaO cells).

As it was published in (Bertran et al. 2009), these TβT-FaO cells show changes in the cytoskeleton, a switch in the expression of proteins from intermediate filaments, a de-differentiation cell state and they are refractory to TGF-β suppressor effects, both in terms of apoptosis or growth inhibition.

In order to explore the metastatic potential of TGF-β-chronically-treated cells, we made a first experiment injecting FaO and TβT-FaO cells subcutaneously in the flank of Balb/c nude mice as described in the Materials and Methods.

We made this first approach methodology because we needed to be able to monitor and analyze the injected skin area visually, otherwise the different parameters could be difficult to check inside the animal. We wondered if a tumor lesion would appear in both or at least one of the cell types implanted once reached the experimental endpoint. In that case, we wanted to check the tumor and also notice if there were differences in the latency, other alterations in the animals or macroscopically differences between the tumors. There were used 9 mice for FaO cells injection and 7 mice for TβT-FaO injection and the experimental period lasted 30 days.

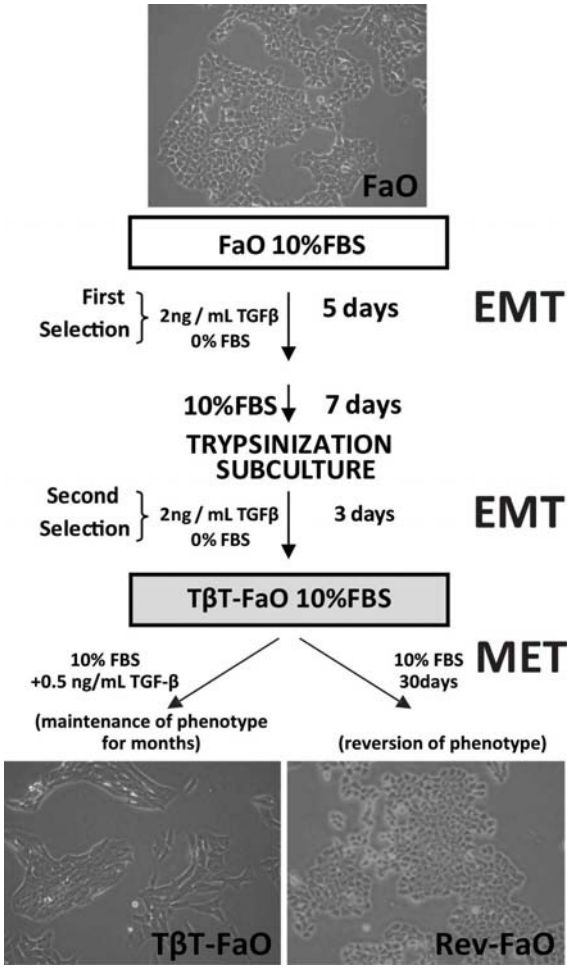
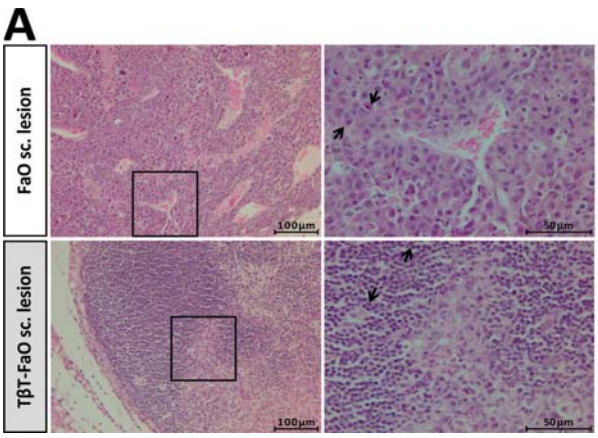


Figure 17. Isolation of FaO rat hepatoma cells that have undergone EMT after chronic treatment with TGF-β. Isolation of FaO rat hepatoma cells that have survived to two cycles of TGF-β treatment, as indicated in the Figure (TβT-FaO, from TGF-β-treated FaO cells). Phenotype is maintained when TGF-β (0.5 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. Phase-contrast photographs displayed the different morphologies of FaO, TβT-FaO and Rev-FaO cells. EMT: epithelial-mesenchymal transition; MET: mesenchymal-epithelial transition.

The experimental implantation showed that both FaO cells (89% (8/9) of animals) and TβT-FaO cells (100% (7/7) of animals) implantation induced lesions in mice. Animals were examined for development palpable tumors and their volumes were calculated as described in Material and Methods. The tumors' time appearance and growth were similar for both cell types as it's indicated in Figure 18B. The tumor latency period and the Tumor Doubling Time (TDT) were calculated as indicated in Figure 18B. At termination there were no lymph nodes, liver or any other organ metastasis in neither cell type-injected mice.



B

	FaO	TβT-FaO
Latency (days)	24.50 ±0.71	19.67 ±2.08
TDT (days)	3.02 ±0.53	2.48 ±1.44

Figure 18. Subcutaneous injection of FaO and TβT-FaO cells induces histological different tumors but with similar doubling time and latency. A. Hematoxylin-Eosin staining of FaO and FaO-TβT cells subcutaneous-induced lesions. Left column 20X magnification, right column 60X magnification of the box area. Arrows show individual cells in both tumors. **B.** Table showing different parameters in both induced tumors. The tumor latency period was determined as the time in days tumors needed to have a detectable volume in each animal. Data are mean ±SD. We decided a minimum of $5mm^3$ to standardize the calculations. The Tumor Doubling Time (TDT) was calculated as the days for each tumor to growth from "x" to "2x" volume, according to its growth date over time. Data are mean ±SD. We decided an initial volume of $5mm^3$ in each tumor to standardize the calculations. The tumors' time appearance and growth were similar for both cell types.

The tumors obtained from FaO and TβT-FaO tumor injection were histological and macroscopically different (Figure 18A). The nuclear-cytoplasmic ratio, known as the size of the nucleus of a cell to the size of the cytoplasm of that cell, was different in both lesions (arrows in Figure 18A). The TβT-FaO-induced lesion display dysplasic hepatocytes with irregular enlarged nuclei (higher nuclear-cytoplasmic ratio) compared to the FaO-induced lesion and that can point out stemness and more aggressive behavior in TβT-FaO tumors.

2.2. Study of the metastatic potential of TGF-β-chronically-treated-FaO hepatoma cells by orthotopic implantation into the spleen.

Taking into consideration the previous results in a subcutaneous injection, we decided to explore deepen in the metastatic potential of TGF-β-chronically-treated cells by inoculating FaO and TβT-FaO cells into the spleen of Balb/c nude mice, as described in the Materials and Methods.

We tested this second approach methodology because successful metastasis depends in part on the interaction of favored tumor cells with a compatible milieu provided by a particular organ environment. We hypothesized that from the spleen tumor cells could

easily gain access to the blood stream and then reach the liver, where they proliferate into tumor colonies because of the liver-specific environment.

In order to avoid any interference from cells or any liver tumor growing into the spleen, we submitted mice to splenectomy two days after injection as described in the Materials and Methods. Here we show the calculations from a representative experiment where 10 mice for FaO cells injection and 16 mice for TβT-FaO injection were used and the experimental period lasted 55 days.

We removed from the statistics calculations shown in Table I the animals that died because of surgery complications or other mice aggressions. Animals were examined twice weekly looking for symptoms of tumors (as described in Material and Methods) and were sacrificed when a tumor was detected. The maximum incidence for FaO injected animals (sacrifice of 50% of mice) was between day 42-44 post-injection and between days 46-55 post-injection for TβT-FaO injected animals (sacrifice of 56.25% of mice). The total mice's sacrifice was performed between days 29-44 for the FaO-injected mice and between days 33-55 for the TβT-FaO-injected mice. At termination mice were killed, tumors were excised, and processed for Hematoxylin and Eosin staining and immunohistochemistry for histopathologic examination. The animals were autopsied and examined for any evident tumor metastasis at distant visceral sites.

All the FaO-inoculated mice presented hepatic metastasis (8/8) and the 92.3% in the case of TβT-FaO-inoculated mice (12/13). A similar percentage of mesenteric metastasis was found but none in the lungs in both groups of mice as it's represented in Table I. The liver metastasis obtained from FaO-injected mice presented a high number of metastatic foci compared with TβT-FaO-injected ones and displayed also differences in the number and size of mesenteric metastasis even thought the percentage of mice is similar. Intrasplenic injections of TβT-FaO cells behave less aggressive with respect to their parental FaO cells, but showed severe anemia.

	ϴ liver metastasis	¥ mesenteric metastasis
FaO Nº of foci	100% (8/8) (+++)	62.5% (5/8) (++)
TβT-FaO Nº of foci	92% (12/13) (+/++)	53.9% (7/13) (+/+++)

Table I. Incidence of experimental mestastasis after intrasplenic inoculation of FaO and TβT-FaO cells. The total mice's sacrifice was performed between days 29 and 44 for the FaO-injected mice and between days 33 and 55 fot the TβT-FaO-injected mice. Results are expressed in percentage of animals that developed metastasis at the time of sacrifice. Mice which died because of surgery complications or other mice aggressions were removed from calculations. ϴ Indicates the incidence of liver metastasis. ¥ indicates the incidence of mesenteric metastasis. The number of metastatic foci in individual mice is indicated by "+". + indicates 1-3 metastatic foci of $\leq 1mm^3$ in size were detected, ++ indicates the presence of 3-10 metastatic foci of $\geq 1,5$ mm^3 in size, +++ indicates the presence of more than 10 metastatic lesions.

Lesions found in FaO-inoculated animals were similar in all the experiments performed. Macroscopically FaO-obtained tumors induced a multifocal hepatocarcinoma in all mice. Otherwise, liver tumor formation derived from intrasplenic injection of TβT-FaO cells displayed heterogeneous lesions mostly unifocal foci, bigger than FaO's ones (Figure 19B). Macroscopically TβT-FaO-obtained tumors were dissimilar among them. Most of the lesions were more irrigated and seemed less differentiated, reminding hepatoblastoma-like tumors (we named these tumors TβT-R for its red lesions). However, white lesions also appeared, reminding hepatocellular carcinoma or cholangiocarcinoma tumors (we named these tumors TβT-W for its white lesions).

FaO-induced liver tumors have multiple clusters surrounded by connective tissue (pointed with non-continuous lines in Figure 19A, upper row) in all the tumor area. These structures were also found in TβT-W liver tumor (also pointed with non-continuous lines, Figure 19A, middle row) in a lowest quantity, collectively with cells polarized in circle, resembling enlarged ductal structures, which are present in all the tumor area (pointed with arrows in Figure 19A, middle row). TβT-R lesion doesn't present any organized structured like the other two types of tumors (see the region inside the non-continuous line in Figure 19A, lowest row), but a large amount of sinusoids (pointed with asterisks in Figure 19A, middle row).

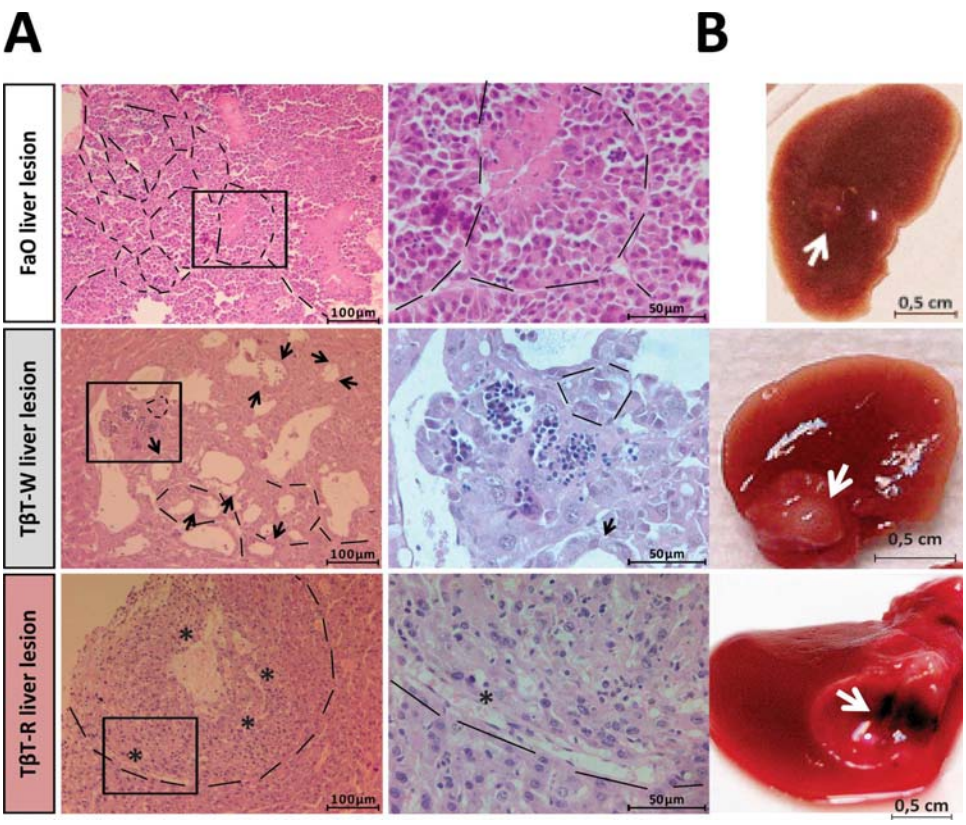


Figure 19. Intrasplenic inoculation of TβT-FaO cells induce macroscopically different tumors compared to the ones obtained from FaO cells inoculation. A. Hematoxylin-Eosin staining of FaO and FaO-TβT cells intrasplenic-induced lesions. FaO-TβT-induced tumors are named as TβT-W for its white appearance and TβT-R for its bloody appearance. Left column 20X magnification, right column 60X magnification of the box area. "-" indicates the presence of clusters surrounded by connective tissue in FaO and TβT-W tumors and the disorganized tumor area in TβT-R lesion. "↖" indicates the presence of enlarged ductal structures in TβT-W lesion. "*" indicates the presence of a sinusoid in TβT-R lesion. **B.** Photographs of the tumors obtained by the different cells inoculation. Arrows point the different macroscopic lesions. **A-B.** Are representative from three experiments.

Results obtained from subcutaneous inoculation of both Fao and TβT-FaO cells didn't show significant differences in the latency or tumor doubling time (Figure 18B), but we wanted to know if there were differences when cells are intrasplenically inoculated. We first focused on the analysis of possible differences in cell proliferation, late apoptosis or phosphorylated Smad2 (hallmark of TGF-β pathway activation).

The assessment of cell proliferation by the detection of Ki67 antigen in neoplastic cell populations has been shown to be of prognostic value. Ki67 antibody labels in the granular components of the nucleolus during late G1, S, G2 and M phases. There is a strong correlation between low or high Ki67 index and low or high grade histopathology of neoplasms (Scholzen and Gerdes 2000). Ki67 immunostaining performed in the different tumors shows that FaO liver tumor has a high proliferative rate compared

with the TβT-FaO-induced ones. Interestingly the TβT-W liver tumor proliferates more than the TβT-R liver tumor (Figure 20A, D).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids. It was originally described by Garvrieli and colleagues (Gavrieli et al. 1992), TUNEL has become one of the main methods for detecting apoptotic programmed cell death. However, for years there has been a debate about its accuracy, due to problems in the original assay which caused necrotic cells to be inappropriately labeled as apoptotic. (Grasl-Kraupp et al. 1995). The method has subsequently been improved dramatically and if performed correctly should only identify cells in the last phase of apoptosis (Negoescu et al. 1998, Negoescu et al. 1996). TUNEL positive cells were detected in each tumor lesion and counted for statistical analysis. TUNEL staining revealed that FaO-derived lesion was widely positive for dying cells whereas staining was greatly reduced in TβT-FaO-obtained lesions (Figures 20B,E). Interestingly TUNEL staining data mostly correlate with Ki67 positive cells stadistics, indicating a compensatory hepatocyte proliferation in the lesions (Figures 20D,E).

P-Smad2 staining was mostly localized in the nucleus (Figure 20C) (although there was also little cytoplasm staining in some cells), with different pattern of staining in the different types of liver tumor examined. FaO-induced tumor displayed weaker staining nuclei with only 30% of total cells positive for p-Smad2 staining. Stain intensity in the nucleus was significantly higher between FaO liver tumors and FaO-TβT-induced tumors (Figure 20F). TβT-W tumor was positive for p-Smad2 staining but mostly displaying a weak staining and there were negative cells whereas in TβT-R lesion we could barely found negative nuclei and there was similar number of high and low staining cells for p-Smad2.

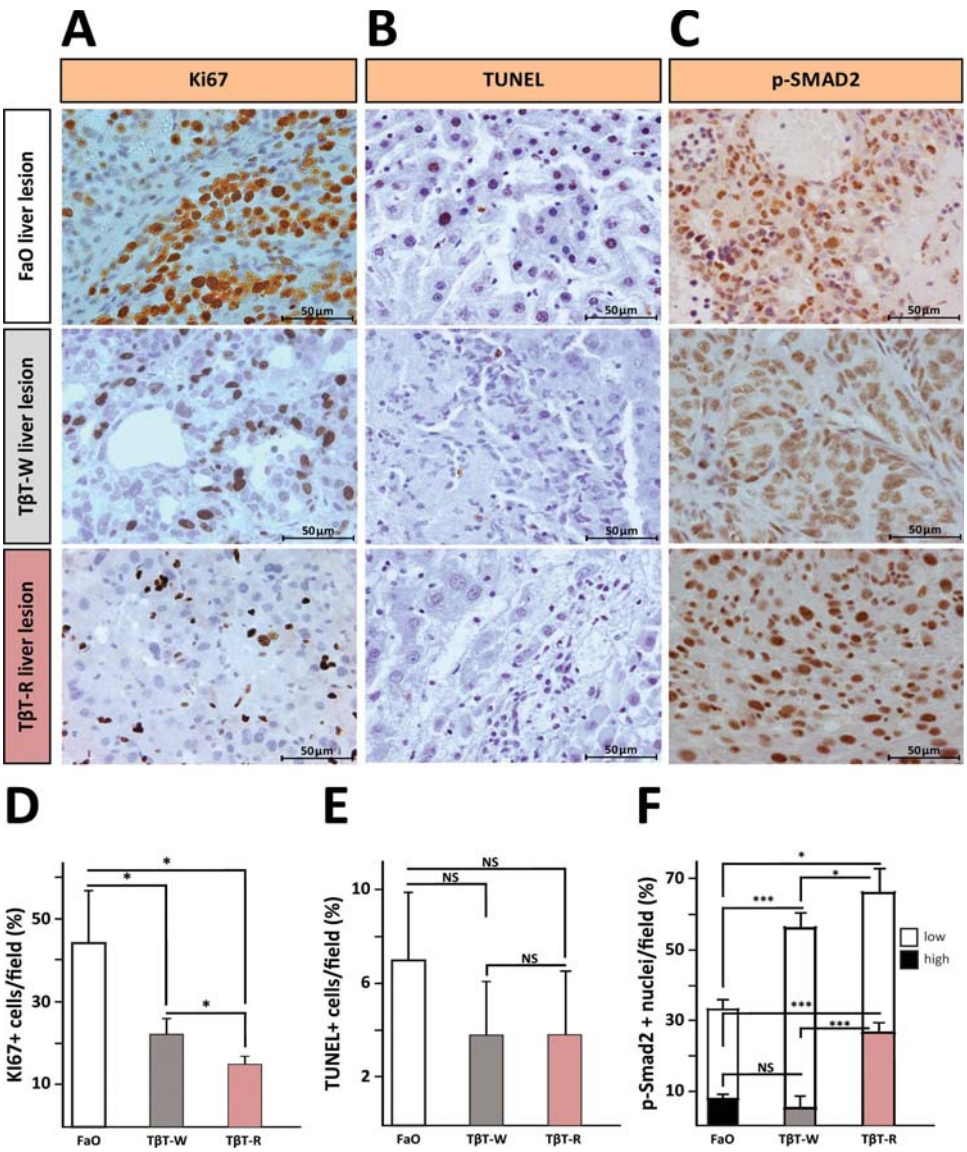


Figure 20. FaO-intrasplenically-induced tumors are more proliferative and have low levels of p-Smad 2 compared to the TβT-FaO-induced ones. A,B,C. Ki67, TUNEL and p-Smad2 immunostaining respectively performed in FaO, TβT-W and TβT-R liver tumors. Representative photographs were taken at 60X magnification and are representative from three experiments. **D,E,F.** Quantification of positive cells for Ki67, TUNEL and p-Smad2 respectively. Low and high in p-Smad2 graph mean weak or strong staining respectively. Data represent the mean± SEM of the number of positive cells in at least ten different fields and they were compared as indicated in the Figure. Student's *T*-test: **P*<0.05; ****P*<0.001; NS: no significant**.**

Smad 7 staining, as a negative regulator of TGF-β signalling, inversely correlates with P-Smad2 in these lesions (results not shown). Data presented correlates with the higher aggressive tumors found in FaO-inoculated mice compared to the TβT-FaO ones, as

inactivation of Smad2 and 3 enhances tumor growth by blockage of autocrine TGF-β negative growth signal (Matsuzaki et al. 2000).

The epithelial to mesenchymal transition has been a topic of interest in our group. Since FaO-TβT cells have undergone EMT *in vitro* after TGF-β-chronic treatment (see Figure 17 for further information) we wanted to know what kind of different tumors these cells could induce in comparison to its parental FaO when intrasplenically injected. Regarding FaO-induced tumors, we wonder whether they would be sensitive to EMT-inducing signals originated from the tumor-associated stroma (including TGF-β signaling) and undergone an EMT process *in vivo*.

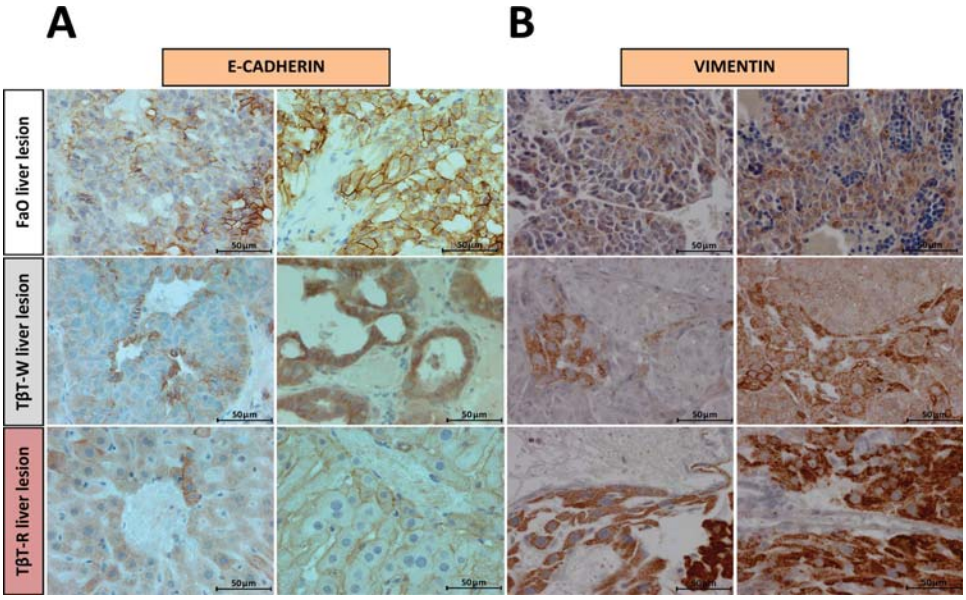


Figure 21. E-cadherin and Vimentin staining in intrasplenic induced tumors. A,B. E-cadherin and Vimentin immunostaining respectively performed in FaO, TβT-W and TβT-R liver tumors. Representative photographs were taken at 60X magnification and are representative from three experiments.

Liver lesions obtained from FaO inoculation displayed a well organized and strong E-cadherin staining as expected (Figure 21A, upper row right column), although there were regions that have lost E-cadherin expression reflecting that EMT can also occur *in vivo* (Figure 21A, upper row left column). Supporting this idea, FaO liver tumor shows weak but positive staining for Vimentin (Figure 21B, upper row) though the original cells FaO didn't display any (Bertran et al. 2009). This change in the expression of E-cadherin to Vimentin could reflect an EMT process in vivo in these cells. TβT-W liver lesion showed E-cadherin expression also well organized and with a strong signal in some ductal structures (Figure 21A, middle row) and displayed a middle intensity Vimentin expression and not in all cells (Figure 21B, middle row). These changes in the adhesion molecules staining could indicate that these cells have differentiated *in vivo*. Liver lesions TβT-R display E-cadherin and Vimentin staining as expected because of the

original cells FaO-TβT: a weak staining for E-cadherin (Figure 21A, lower row) and a strong expression of Vimentin (Figure 21B, lower row).

Next we explored possible changes in Cytokeratins in these liver tumors as they're a valuable tool for classifying liver cells according to its maturity and lineage. Adult hepatocytes express both Cytokeratin 8 and 18 while mature cholangiocytes express Cytokeratin 7, 8, 18 and 19 (Shiojiri 1997). Cytokeratin 7 is also expressed in liver stem cells (Libbrecht and Roskams 2002). Cytokeratin 18 and 19 are both expressed in hepatoblasts. Cholangiocytic differentiation maintains expression of both Cytokeratin 18 and Cytokeratin 19, with predominance of Cytokeratin 19. In contrast, hepatocyte differenciation represses Cytokeratin 19 expression only maintaining Cytokeratin 18. Furthermore also SPARC it's been pointed as a marker of stemness in neural stem cells (Huang et al. 2008).

In Figure 22, upper row, the immunostaining performed in the FaO liver tumor indicated that was negative for Cytokeratin 7, Cytokeratin 19 and SPARC staining (see Figure 12 to see data of parental FaO cells) and positive for Cytokeratin 18 as corresponds to a mature hepatocyte. Both TβT-W and TβT-R lesions showed positive staining for Cytokeratin 19. The TβT-W tumor (see Figure 22, middle row) was almost negative for Cytokeratin 7 and positive for Cytokeratin 18 and SPARC in delimitated areas in the parenchyma. In contrast, the TβT-R lesion (see Figure 22, lowest row) was positive for Cytokeratin 7 (indicating a possible dedifferentiated phenotype), Cytokeratin 18 and SPARC.

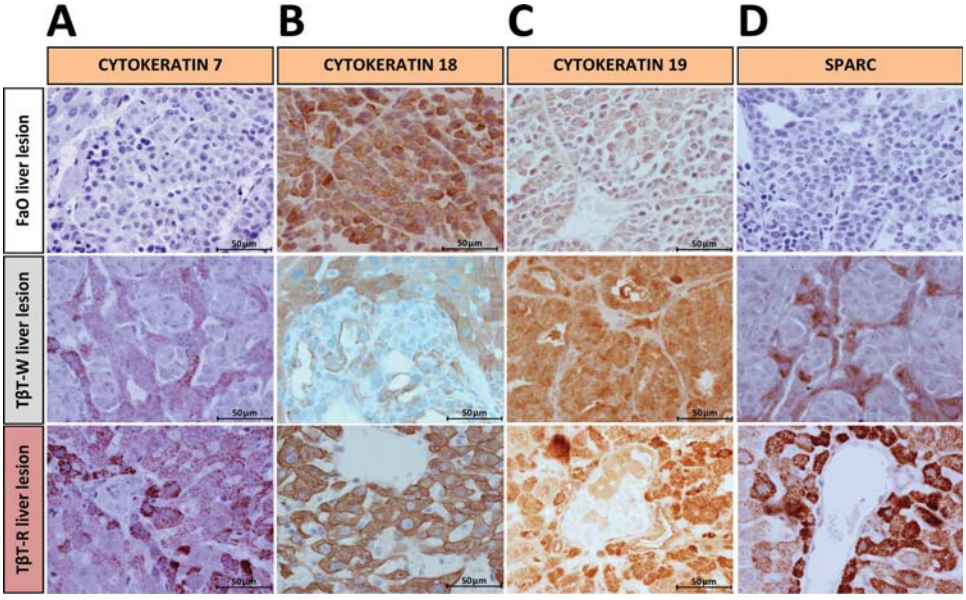


Figure 22. Intrasplenic injection of TβT-FaO cells generated lesions with bile duct characteristics and lesions with a dedifferentiated phenotype. A-D. Cytokeratin 7, 18, 19 and SPARC immunostaining respectively performed in FaO, TβT-W and TβT-R liver tumors. Representative photographs were taken at 60X magnification and are representative from three experiments.

2.3. Isolation and characterization of intrasplenic-tumor-obtained cell lines.

To analyze the relevance of the results obtained in the immunostaining studies performed in the liver tumors and to better characterize the resulting phenotype, we decided to obtain primary cultures from the different lesions as described in the Materials and Methods. The cell culture obtained from FaO-induced tumor was named FaO-D, the cell cultures obtained from TβT-FaO-induced lesions were named TβT-W-D (corresponding to TβT-W white lesions) and TβT-R-D (corresponding to TβT-R red lesions).

We first observed the appearance of these cells in culture, finding that FaO-D were similar to the parental FaO we inoculated in the animal (Figure 22A). The two cell cultures obtained from TβT-FaO intrasplenic inoculation presented both morphology similar to the original cell line, but the TβT-W-D cell culture formed ductal-like structures and some cells formed parenchymas while the TβT-R-D cell culture was similar to the TβT-FaO parental cell line, with cells growing isolated and with a typical fibroblastic appearance (Figure 23A).

We wanted to corroborate if the observations done with the ki67 staining (Figure 20A,D) corresponding to the proliferation ratio of the lesions was correlated with in vivo growing. As it's demonstrated in Figure 23B, the FaO-D cell culture has a higher growing rate compared to the TβT-W-D and TβT-R-D cell cultures which directly correlates with ki67 staining in the liver tumors.

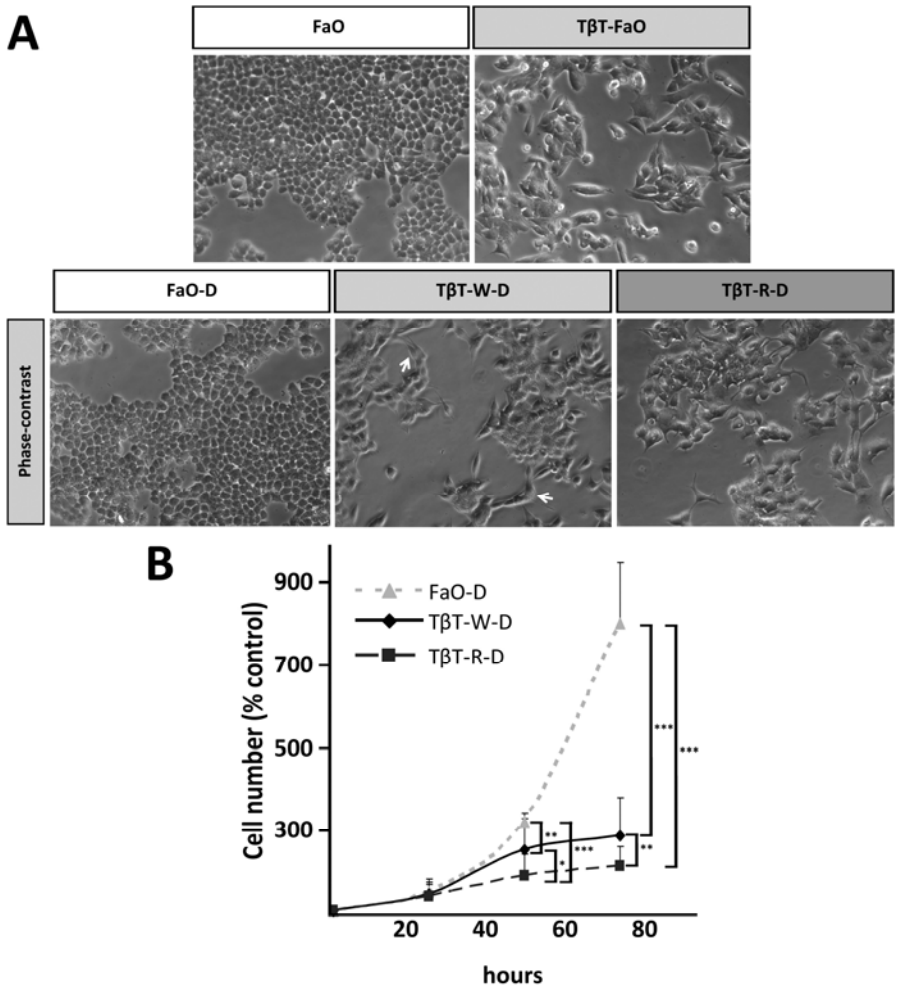


Figure 23. The FaO-D cell culture has a higher growing rate compared to the TβT-W-D and TβT-R-D cell cultures. **A.** Phase-contrast photographs display the different morphologies of FaO and TβT-FaO cell lines (upper row) and FaO-D, TβT-W-D and TβT-R-D cell cultures (lower row). Arrows in TβT-W-D cell culture pointed ductal-like structures. **B.** Number of viable cells analysed by crystal violet staining. Results are expressed as percentage of the initial number of cells and are mean ± S.E.M. of three independent experiments. Student's *T*-test: ***P*<0.01; ****P*<0.001.

Next, we wondered whether living and forming a tumor in the liver environment and tumor-associated-stroma would affect its ability to respond to TGF-β in terms of apoptosis. We submitted the cell cultures we obtained from the liver lesions to TGF-β (Figure 24) and interestingly both TβT-W-D and TβT-R-D were refractory to TGF-β-induced apoptosis as the parental TβT-FaO but the FaO-D cell culture induced Caspase-3 activity as the parental FaO cell line does (Bertran et al. 2009).

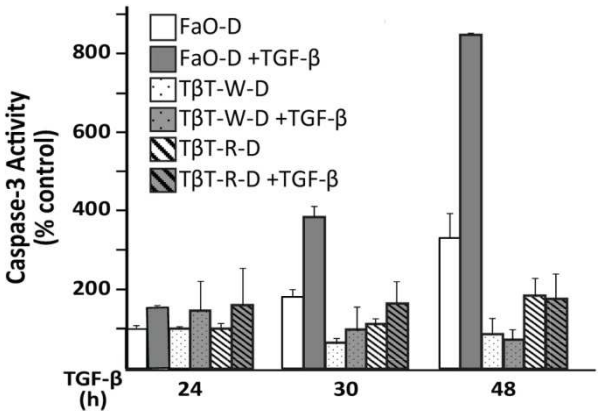


Figure 24. The FaO-D cell culture induces Caspase-3 activity in response to TGF-β treatment. Caspase 3 activity was analyzed by fluorimetry and presented as the percentage of the cell culture controls at 24 hours. Results are mean ± S.E.M. of two independent experiments.

Taking into consideration the results obtained in the tumors immunostaining in Figure 21, we wanted to further analyze the expression of the typical EMT markers E-cadherin and Vimentin. The cell culture FaO-D displayed a well organized and strong E-cadherin staining as the FaO cell line does (Figure 25A upper row) although has weak but positive staining for Vimentin in some areas of the parenchyma (Figure 25B upper row) though the original cells FaO didn't display any (Bertran et al. 2009). FaO-D cell culture displayed F-actin cytoskeleton in a pericellular localization as the original cells FaO (Figure 25C upper row).

In agreement with Figure 21 results, the TβT-W-D cell culture showed E-cadherin expression well organized and with a strong signal but not uniformly (Figure 25A middle row) and displayed Vimentin expression in some cells (Figure 25B middle row). The F-actin staining revealed a well-established parenchyma in this TβT-W-D cell culture, more similar to FaO-D than to TβT-R-D cell culture (Figure 25C, middle row). Moreover the TβT-R-D cell culture displayed a weak and pretty uniform staining for E-cadherin (Figure 25A lower row) and a strong expression of Vimentin (Figure 25B lower row) and distribution of F-actin into stress fibers as expected out of the original cells FaO-TβT (Bertran et al. 2009).

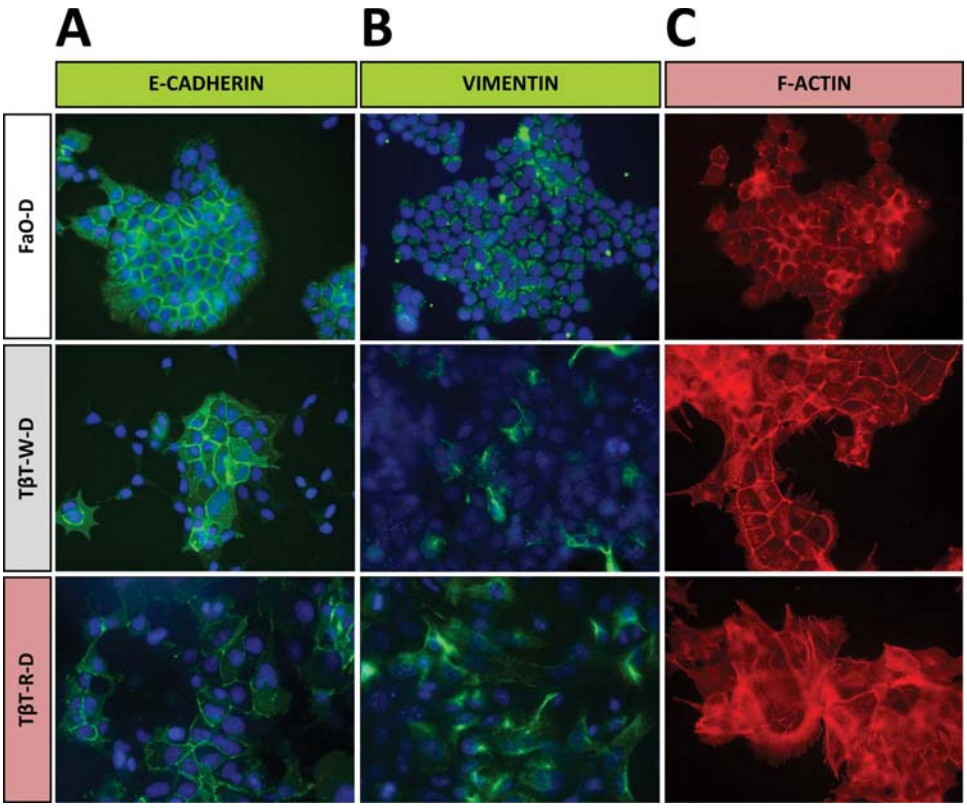


Figure 25. F-Actin, E-cadherin and Vimentin staining in cell cultures obtained from intrasplenic induced tumors. A,B. E-cadherin and Vimentin immunofluorescence respectively performed in FaO-D, TβT-W-D and TβT-R-D cell cultures. **C.** F-actin filaments were stained with rhodamine-conjugated phalloidin in all the cell cultures. Representative photographs were taken at 60X magnification and are representative from three experiments

To confirm the results observed in the immunostaining studies performed in Figure 22, we explored Cytokeratins expression in the obtained-cell cultures. The FaO-D cell culture showed no staining either for Cytokeratins 7 or 19 (Figure 26A,C upper row) and positive staining for Cytokeratin 18 (Figure 26B upper row) as the parental FaO cell line (Bertran et al. 2009). Both TβT-W-D and TβT-R-D cell cultures showed positive staining for Cytokeratin 19 (Figure 26C middle and lower rows). Confirming the previous results in liver lesions, TβT-W-D cells were almost negative for Cytokeratin 7 and positive for Cytokeratin 18 in some cells (Figure 26A,B middle row). In contrast, TβT-R-D cells (Figure 26A,B lowest row) were strongly positive for Cytokeratin 18, but also showed expression of Cytokeratins 7 and 19.

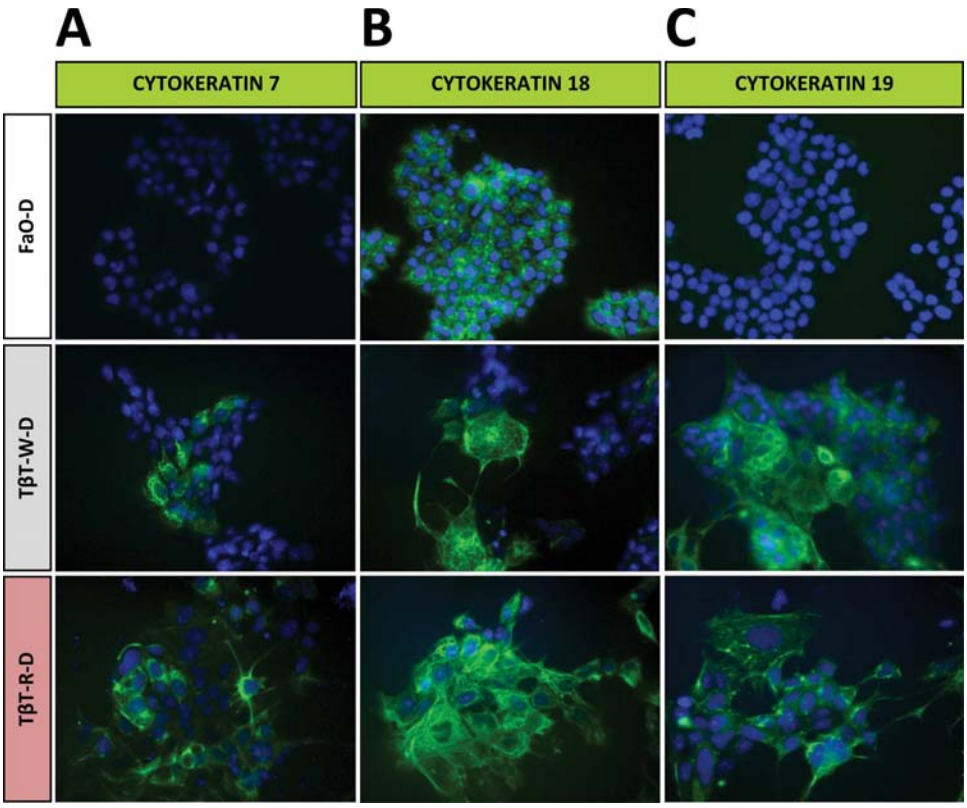


Figure 26. TβT-FaO-intrasplenic-inoculation-derived cell cultures present different pattern of Cytokeratins. A-C. Cytokeratin 7, 18 and 19 immunofluorescence respectively performed in FaO-D, TβT-W-D and TβT-R-D cell cultures. Representative photographs were taken at 60X magnification and are representative from three experiments.

Taking into consideration the results obtained by our group in the recent years (Bertran et al. 2009), we decided to explore the differentiation state of all this obtained cell cultures compared to the parental cell lines and corroborate the results obtained in the immunofluorescence studies.

Figure 27. Analysis of EMT and hepatic differentiation genes in FaO-D, TβT-W-D and TβT-R-D compared to FaO and FaO-TβT. Transcript levels of *Hnf4a*, Foxa2(*Hnf3θ*), *Epcam, c-Kit, Krt7, Krt19* (Cytokeratins 7 and 19) , *Snai2* (slug), *Snai1*, *Vim* (Vimentin) and *Sparc* were studied in FaO, FaO-TβT, TβT-W-D, TβT-R-D and FaO-D by semiquantitative PCR in order to determine changes related to the EMT process and differenciation state. *18S* levels were used as normalization control. Data are representative of three experiments.

As it's indicated in Figure 27, FaO-TβT parental cells and TβT-W-D and TβT-R-D cell cultures presented a de-differentiated state. These cells had lost the expression of the Hepatocyte Nuclear Factor Hnf4α, characteristic of terminally differentiated liver cells without affecting Hnf3β, which is expressed in earlier stages of liver differentiation. Interestingly, Epcam (Epithelial Cell Adhesion Molecule) a marker for hepatic stem cells (Schmelzer et al. 2006) was found in all FaO-TβT, TβT-W-D and TβT-R-D. Another stem cell marker c-Kit, which is highly expressed in cholangiocytes, was mainly detected in TβT-W-D cells (see also Figure 28A). As a marker for bile duct epithelium and cholangiocytic differentiation, we found Cytokeratin 19 expression in TβT-W-D which we associated to a cholangiocytic phenotype, although it was also presented in the TβT-R-D culture at lower intensity.

The EMT process induced the expression of the transcription factor Snail and Vimentin in all FaO-TβT, TβT-W-D and TβT-R-D. Interestingly, Slug (another important transcription factor involved in EMT) is expressed in FaO-TβT, TβT-R-D and FaO-D (which could explain the presence of the mesenchymal marker Vimentin in the liver lesion immunostaining and also in FaO-D immunofluorescence). As Slug is an important factor for *SPARC* expression and SPARC it's been related to stemness, its expression correlates with a big SPARC induction in FaO-TβT and TβT-R-D cells.

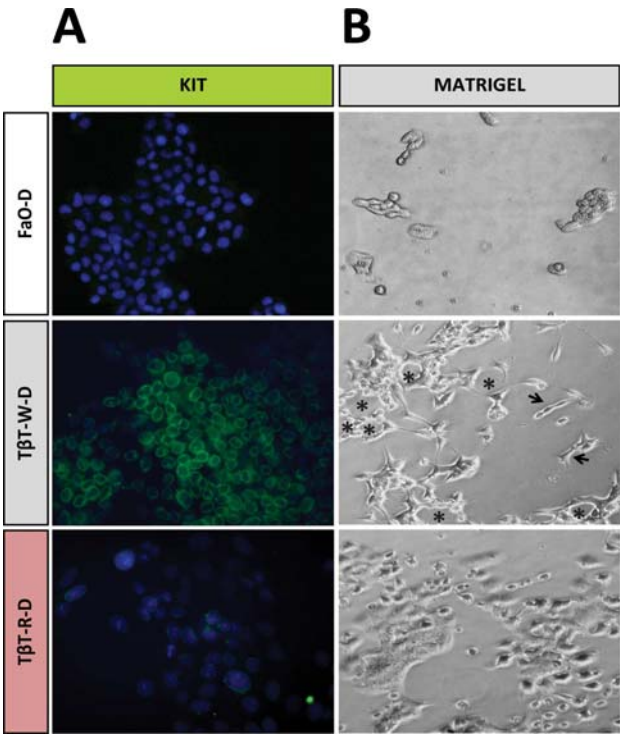


Figure 28. TβT-W-D express c-kit receptor and form ductular structures when cultured in Matrigel. A. c-kit immunofluorescence respectively performed in FaO-D, TβT-W-D and TβT-R-D cell cultures. Representative photographs were taken at 60X magnification and are representative from three experiments. **B.** Phase-contrast photographs displayed the different morphologies of FaO-D, TβT-W-D and TβT-R-D cells when placed on Matrigel. Arrows pointed short chains of cells that evolucionate to tubular estructures resulting in duct-like structures (pointed as asterisks). Representative photographs from two experiments.

To corroborate the results obtained from semi-quantitative PCR, we performed a c-kit immunofluorescence in FaO-D, TβT-W-D and TβT-R-D. Confirming the previous results, we only observed staining in the TβT-W-D culture (Figure 28A).

We wanted to answer an important point to confirm that the TβT-W-D cells were behaving as cholangiocytes. As the cholangiocytes have the ability to form ductular structures when placed in Matrigel (Ader et al. 2006) we wanted to check the possible morphological changes as described in the Materials and Methods. As it's shown in (Figure 28B) only the TβT-W-D cells were capable of forming tubular and finally ductular structures in response to culture on Matrigel.

In summay, the intraesplenic inoculation of FaO rat hepatoma cells gave rise to tumors that resemble HCC while chronic *in vitro* TGF- β treatment of FaO cells changed their tumorigenic potential. Tumor growth was similar, but phenotype of lesions reflected a stem-like phenotype which provokes the appearance of less differentiated tumors (hepatoblastomas) or transdifferentiation to a different liver tumor lineage (cholangiocarcinoma).

VIII. DISCUSSION

1. Regulation by TGF-β of EMT-related genes that could control apoptosis in hepatocytes: Role of Snail and SPARC in TGF-β suppression function.

The complex and sometimes contrasting signals induced by TGF-β in epithelial cells (Massagué, 2008; Zavadil and Bottinger, 2005; Heldin et al., 2009) make it difficult to understand its specific role in tumour progression. Indeed, its influence might even be cell context-specific and dependent on the extracellular environment.

In the case of the liver, recent findings have offered new insights into the role of TGF-β in human hepatocarcinogenesis. Indeed, its clear tumor suppressor role evident at early stages is converted to a tumor promoter function at advanced stages (Massagué, 2008). Liver cancer cells become resistant to TGF- β -mediated cell death, and they become capable of undergoing EMT and acquiring invasive properties (Sanchez et al. 1999, Valdes et al. 2002, Valdes et al. 2004, del Castillo et al. 2006, Caja et al. 2007). Accordingly, liver tumors expressing an early TGF-β signature (suppressor genes) have a less invasive phenotype and tumour recurrence when compared with those that express late TGF-β-responsive genes (anti-apoptotic and metastatic) (Coulouarn et al., 2008). Thus, it is crucial to understand the mechanisms that permit liver cancer cells to escape from the suppressive effects of TGF-β, both to understand tumor progression and to design therapies to block the pro-tumorigenic effects of TGF- β in human hepatocellular carcinoma.

1.1. Effects of Snai1 downregulation in the TGF-β induced apoptosis in liver cells. Relevance in human liver tumorigenesis.

The results presented demonstrate that immortalized neonatal murine hepatocytes (MH) undergo an epithelial to mesenchymal transition (EMT) process in response to prolonged expositions to TGF-β (Figure 1,2). In the liver the TGF-β treatment selects a subpopulation that become refractory to its tumor-suppressor effects and undergoes EMT as we have reported in fetal rat hepatocytes (Valdes et al. 2002) and rat hepatoma cells (Caja et al. 2007).

Previous results in our group showed that adult rat hepatocytes failed to induce Snail and to undergo EMT and do not re-organize F-actin in response to TGF-β. This lack of response to TGF-β in actin re-organization in adult hepatocytes might be also related to their inability to activate EGFR (Caja et al. 2007). The mechanism that prevents *Snai1* upregulation in non-transformed adult hepatocytes could be the absence of activated Ras, which is crucial for the TGF-β-mediated induction of *Snai1* expression and EMT in renal cells as well as in hepatic cell lines (Gotzmann et al. 2002, Peinado et al. 2003, Horiguchi et al. 2009, Grande et al. 2009). Thus it's been demontrated in cancer

pancreatic cell lines that Ras and TGF-β-Smad signaling selectively cooperate in the induction of Snail, which occurs in a Smad-dependent manner, but independently of phosphorylation at the linker region of R-Smads by Ras signaling (Horiguchi et al. 2009). Interestingly, forced expression of Snail1 is sufficient to induce EMT in non-transformed adult hepatocytes (Franco et al. 2010). And here we show that *Snai1* does indeed play an important role in regulating this phenotypic transition as its silencing prevents the TGF-β-mediated E-cadherin loss and EMT (Figure 4).

We also have analyzed the importance of *Snai1* expression in the apoptotic response to TGF-β in MH, finding that *Snail1* down-regulation significantly enhances the apoptotic response to TGF-β of liver cells (Figure 5), which indicates that Snail overcomes the cell death induced by TGF-β. These results indicate that Snail1 coordinates the EMT with cell survival signals in the liver, which is reminiscent of situations taking place during embryonic development (Barrallo-Gimeno and Nieto 2005). In agreement with our results, recently it has been published that Snail1 promotes resistance to apoptosis, an effect associated to PTEN gene repression and Akt stimulation, by direct enhancing the binding of Akt2 to the E-cadherin promoter (Villagrasa et al. 2011).

Conversely, Snail activation confers full resistance to TGF-β-induced apoptosis in immortalized mouse hepatocytes and in adult hepatocytes obtained from Snail1-ER transgenic mice. Snail1 can also decrease cell death in cultured liver cells when compared with control cells, indicating that Snail not only protects from the death induced by TGF-β (Franco et al. 2010). Indeed, Snail family members are known to confer resistance to the cell death induced by the removal of survival signals, apoptotic stimuli, gamma radiation and genotoxic stress (Inoue et al. 2002, Perez-Losada et al. 2003, Vega et al. 2004, Kajita et al. 2004, Martinez-Alvarez et al. 2004, Vitali et al. 2008).

It is well known that EGF impairs TGF-β-induced apoptosis in hepatocytes and hepatoma cells by inhibiting activation of the caspase cascade (Herrera et al. 2002, Herrera et al. 2001a, Shima et al. 1999b) and its inhibition enhances TGF-β-induced apoptosis (Sancho et al. 2009). One of the EGFR ligands, TGF-α, might also protect from apoptosis in murine hepatocellular carcinomas through the activation of nuclear factor-κB (NF-κB) (Cavin et al. 2005). Besides, HB-EGF is a potent inducer of tumor growth and angiogenesis (Ongusaha et al. 2004) and it has been proved to be an early response gene to chemotherapy and contributes to chemotherapy resistance (Wang et al. 2007).

Previously we have published that TGF-β, additionally to its pro-apoptotic activity, also induces anti-apoptotic signals. TGF-β is able to transiently activate PI3K/Akt (Valdes et al. 2004) and mediates carcinogenesis by transactivation of the EGFR pathway in FaO rat hepatoma cells (Caja et al. 2007). Actually, there is no doubt that phosphatidylinositol 3-kinase (PI3K) pathway interferes with the apoptotic effect of TGF-β in different cell types. Signaling pathways, such as Ras/PI3K, which can be activated by growth factors or hormones like insulin that signal via receptor tyrosine

kinases (RTK), protect cells from TGF-β mediated apoptosis by enhancing the activity of Akt/PKB (Chen et al. 1998). TGF-β can induce itself anti-apoptotic signals though the activation of Akt (Song et al. 2006, Wilkes et al. 2005, Valdes et al. 2004), which in turn can prevent TGF-β-induced apoptosis. It's also have been reported that the early activation of Akt can be related to the TGF-β ability of transactivating c-Src and EGF pathways (Park et al. 2004, Murillo et al. 2005). The activation of c-Src by TGF-β1 is EGFR dependent and is required for full Akt phosphorylation and therefore cell survival in rat hepatocytes (Murillo et al. 2005). We studied in depth which could be the intracellular signals involved in the Snail-mediated survival in MH and we have demonstrated in Figure 6 that murine hepatocytes induce the expression of EGFR ligands and *Snai1* downregulation impairs this effect. This result correlates with the inhibition of Akt, Erks and c-Src family phosphorylation promoted by TGF-β after *Snai1* silencing (Figure 6). The blockage of typical survival pathways after *Snai1* downregulation indicates that Snail1 plays an important role in the survival signals mediated by TGF-β in hepatocytes.

There are evidences showing that the sensitivity to apoptosis induced by TGF-β is regulated by the relation between Akt/PKB and Smads and the ratio of Smad3 protein over Akt/PKB levels defines whether an epithelial cell undergoes apoptosis in response to TGF-β. According to one model, Akt directly interacts with unphosphorylated Smad3 to sequester it outside the nucleus, preventing its phosphorylation and nuclear translocation. This prevents TβRI-mediated phosphorylation of Smad3 and apoptosis (Conery et al. 2004, Remy et al. 2004). Remy and colleagues (Remy et al. 2004) suggested a very simple mechanism by which growth and anti-apoptotic signalling, mediated by PI(3)K–PKB, towards TGF-β signalling by inducing an interaction between PKB and Smad3, thus reducing the pool of Smad3 available for TGF-β signalling. This mechanism is unique in being independent of PKB kinase activity, unlike parallel pathways through which PKB protects against apoptosis (Kim et al. 2002a, Wildey et al. 2003). According to another model, Akt/PKB inhibited Smad3 phosphorylation via its downstream effector, mammalian target of Rapamycin (mTOR), in a kinase-dependent manner (Song et al. 2006). Recently it has been published a mechanism for Akt activation by TGF-β through the induction of two microRNAS , both which target PTEN (phosphatase and tensin homologue), an inhibitor of Akt activation, in glomerular mesangial cells. They demonstrate that Akt blocks phospho-activation of Smad3 by an Akt kinase-dependent mechanism through mTOR and also blocks TGF-β signals downstream of Smad3 activation, but through a mechanism that does not require the kinase activity of Akt or mTOR. This is the first direct evidence for roles of Akt kinase and mTOR as suppressors of Smad3 phosphoactivation (Kato et al. 2009).

Furthermore, the ratio of Smad3 to Akt correlates with the sensitivity of cells to TGF-β-induced apoptosis. Alteration of this ratio changes the apoptotic, but not the growth-inhibitory, responses of cells to TGF-β (Conery et al. 2004). We investigated the Smad signalling in our MH model finding that Smad3 phosphorylation was advanced in MH

cells with *Snai1* knock-down (Figure 7). Interestingly, Smad2, which has been reported to be implicated more in hepatocyte growth inhibition and is required for stable epithelial phenotype of primary hepatocytes *in vitro* (Ju et al. 2006) showed a similar phosphorylation regardless Snail is or not silenced (Figure 7).

Previous results in our group indicate that TGF-β can induce anti-apoptotic signals in fetal hepatocytes (Murillo et al. 2005), FaO rat hepatoma cells (Caja et al. 2007) and human liver tumor cells (Caja et al. 2009), mediating an increase in the intracellular content of anti-apoptotic proteins of the Bcl-2 family, Bcl-xL and Mcl1. TGF-β also induces represion of the anti-apoptotic member Bcl-xL and induces mitochondrial-dependent cell death as we (Herrera et al. 2001b) and other groups (Chipuk et al. 2001) have proved. It has been demonstrated that overactivation of the TGF-β signal in chronic lymphocytic leukemia B cells leads to apoptosis by a mitochondrial-dependent pathway: Bcl-2 and Bcl-xL levels decreased whereas the levels of Bim and Bmf increased (Romano et al. 2008). In other models TGF-β also overexpresses pro-apoptotis BH3-only proteins like Bim and Bmf by a mechanism dependent of Smad4 and p38 (Ramjaun et al. 2007). Bim and Bmf are activators of Bax-Bak (Wildey et al. 2003), which are pro-apoptotic Bcl-2 members that are the downstream effectors controlling the mitochondria-dependent cell death program. The overexpression of Bax and Bak leads to an increase in cell susceptibility to apoptotic signals (Karbowski et al. 2006). Moreover, downregulation of Bax and Bak gene expression with siRNA resulted in an effective reduction of erlotinib-induced cell death in non-small-cell lung cancer (Ling et al. 2008). Our results indicated that downregulation of *Snai1* produces an increase and earlier up-regulation of the levels of *Bim* and *Bmf* (pro-apoptotic members of Bcl-2 family) and a decrease in the expression of *Bcl-xL* and *Mcl1* (anti-apoptotic members) in murine hepatocytes. This expression pattern correlated with a significant enhancement in the percentage of cells showing the active conformational form of Bax or Bak (Figure 8). All these results together indicate an important role of *Snai1* in the regulation of the ratio of the pro-apoptotic versus anti-apoptotic members of Bcl-2 family and an important role in the prevention of mitocondrial-apoptosis induced by TGF-β in hepatocytes.

An important aspect in the metastatic spreading of cancer cells is the ability to survive in the blood or lymph stream, in contrast to non-metastatic adhering cells, which undergo apoptosis shortly after loss of cell– substratum interaction (Chambers et al. 2002). Anoikis (or cell-detachment-induced apoptosis) is a self-defense strategy that organisms use to eliminate 'misplaced' cells, e.g. cells that are in an inappropriate location. Occasionally, detached or misplaced cells can overcome anoikis and survive for a certain period of time in the absence of the correct signals from the extracellular matrix (ECM). Cells which are able to adapt to their new environment have probably become anchorage-independent, which is one of the hallmarks of cancer cells. Anoikis resistance and anchorage-independency allow tumor cells to expand and invade adjacent tissues, and to disseminate through the body, giving rise to metastasis.

Tumor cells have developed a variety of strategies to bypass or overcome anoikis. Some strategies consist of counteract the negative effects of anoikis induction by hyperactivating survival and proliferative cascades. It has been proved that migfilin, an integrin-proximal adaptor protein, interacts with Src and contributes to cell-ECM-mediated survival signaling (Zhao et al. 2009).

In addition, several key regulators of EMT are also involved in survival signaling and anoikis resistance, such as nuclear factor κB (NF-κB), Snail and Twist (Guadamillas et al. 2011). Fischer and colleagues have shown that active β-catenin (its nuclear accumulation it's important in EMT processes) protects malignant hepatocytes from anoikis, which provides a pre-requisite for the dissemination of carcinoma (Fischer et al. 2007). Slug it's been proved to be essential for Notch-mediated repression of E-cadherin, which resulted in β-catenin activation and resistance to anoikis in breast cancer (Leong et al. 2007). It has been reported that Snail, suppresses cell death by inhibiting Caspase-3 and by activating the pro-survival PI3K–Akt pathway (Barrallo-Gimeno and Nieto 2005, Vega et al. 2004).

It has been reported that Bax translocation and activation it's regulated by p38MAPK during mammary epithelial cell anoikis (Owens et al. 2009) and Inhibition of Bim expression by siRNA also decreased apoptosis of mammary cells during anoikis (Reginato et al. 2003). These results directly linked with the up-regulation of Bim and Bax due to Snai1 knock-down we reported (Figure 8) that leads to a less resistance to anoikis in our model of murine hepatocytes (Figure 9). In conclusion, *Snail1* can also control hepatocyte adhesion and confer resistance to the apoptosis induced by loss of contact (like death by anoikis). This effect confirms and expands previous data showing that Snail1 regulates cell attachment to the extracellular matrix (Haraguchi et al. 2008) and with recent data supporting a role for a Twist-Snail axis in the TrkB-induced EMT and anoikis resistance in rat intestinal epithelial cells (Smit et al. 2009).

As a consequence of the failure of liver carcinoma cells to respond to the death-promoting effect of TGF-β, and given their ability to respond in terms of undergoing EMT, hepatoma cells overcome the TGF-β tumour-suppressor effects. We show that both hepatocellular carcinoma cell lines, Hep3B and SK-Hep1, *Snail1* downregulation restores the capacity of cells to respond to TGF-β by undergoing apoptosis, the most important role of TGF-β in controlling hepatocellular carcinoma progression (Figures 10,11). Indeed, Smad3, a physiological mediator of TGF-β-tumour-suppressor activity, functions by repressing Bcl-2 expression and inducing apoptosis (Yang et al. 2006b). Interestingly, *Snail1* acts in an opposite way by increasing *Bcl-xL* and *Mcl1* expression. As Smad3 is also necessary for the TGF-β-induced Snail1 expression (Sato et al. 2003), the final cellular response and perhaps hepatocellular carcinoma progression might depend on the equilibrium between the activation of these two Smad3 targets, Bcl-2 and Snail1.

An interesting conclusion that might be extrapolated from these results is that there is a coordinated regulation between the genetic programs that induce migratory and invasive properties and those that mediate apoptosis and growth, which would confer to the cells a clear advantage to undergo metastatic processes. According to this, a better knowledge of the molecular mechanisms that mediate TGF-β-induced migration and invasion will open new expectatives to effectively exploit the TGF-β system in new therapeutic approaches to cancer.

This resistance promoted by Snail1 confers to the cancer cell a selective advantage to produce and/or activate TGF-β and promote additional carcinogenic processes, through the activation of stromal fibroblasts and inhibiting the antitumor immune response. This suggests that a reactivation of developmental genes such as Snail1 in liver tumor cells contributes to the pro-tumourigenic role of TGF-β.

1.2. Analysis of SPARC expression in immortalized neonatal murine hepatocytes and FaO rat hepatoma cell fire. Effects of SPARC downregulation in the TGF-β-induced EMT and apoptosis in FaO rat hepatoma cells.

SPARC has been clearly implicated in tumor development, but published reports suggest diverse and contradictory functions as either a tumor suppressor or a pro-invasive protein depending on the cell-type and the microenvironment context. SPARC shows over expression in many epithelial malignancies like in prostate, lung or intestine (Clark and Sage 2008), and can promote metastasis (Minn et al. 2005). In melanoma, its expression correlates with aggressiveness and adverse clinical outcome (Massi et al. 1999). Indeed, it has been recently demonstrated that SPARC is involved in the acquisition of mesenchymal traits that contribute to melanoma dissemination (Girotti et al. 2011). It's also been reported the presence of SPARC in the histologically normal but genetically altered tissue margins of human breast carcinomas (Trujillo et al. 2011).

The role of SPARC in HCC, cirrhosis, fibrosis or in normal liver has been controversial. On one hand, SPARC mRNA is absent in extracts from freshly isolated hepatocytes and highly expressed in the fibrotic liver, associated with activated HSC expression (Frizell et al. 1995). In correlation, its down regulation by adenoviral expression of an antisense SPARC attenuates liver fibrosis (Camino et al. 2008) and SPARC downregulation attenuates the profibrogenic response of hepatic stellate cells induced by TGF-β1 and PDGF (Atorrasagasti et al. 2011). These studies are in agreement with the finding of high protein levels in HCC compared with the adjacent non tumor tissues (Luo et al. 2006) and the correlation with succeptibility to HCC (Segat et al. 2009) . On the other hand, a recent study has shown that overexpression of SPARC in HCC cell lines (HepG2, Hep3B and Huh7) results in a reduced tumorigenicity partially through the induction of mesenchymal-to-epithelial transition (MET) (Atorrasagasti et al. 2010).

In our cellular liver models, only the tumoral FaO hepatoma cell line but not the fetal hepatocytes are able to up-regulate SPARC in the TGF-β driven EMT (Figures 1, 12), which associates TGF-β-induced SPARC to liver malignancy. Indeed, as it's demonstrated in Figure 14, SPARC depletion attenuates TGF-β-induced EMT in FaO rat hepatoma cells, which is in agreement with the notion that SPARC contributes to acquire the mesenchymal phenotype and migratory abilities of epithelial tumor cells as it occurs in melanomas (Robert et al. 2006, Smit et al. 2007, Girotti et al. 2011). The association between induction of SPARC and EMT has been reported in different models and cell types as in Sarcomatoid renal cell carcinoma (Conant et al. 2011), endometrial carcinosarcoma (Castilla et al. 2011) or metastatic oral tongue squamous cell carcinoma (Vered et al. 2010). The EMT induced by Snail1 is also associated with the strong induction of SPARC (Moreno-Bueno et al. 2009, Moreno-Bueno et al. 2006).

Although it has been suggested that *Snai1* directly regulates the expression of SPARC in melanoma (Kuphal et al. 2005) we didn't find changes in SPARC levels after *Snai1* knock-down (results not shown) in FaO rat hepatoma cells. Conversely, we did find a decrease in *Snai1* expression levels in SPARC depleted cells (Figure 16). This might result from a cross-talk between TGF-β and SPARC as it has been demonstrated in other systems such as mesangial cells (Francki et al. 1999, Francki et al. 2004), or in a rat model of glomerulonephritis (Bassuk et al. 2000) Indeed, it has been proposed a reciprocal, autocrine regulatory feedback loop between SPARC and TGF-β1 signaling in epithelial mammary cells (Schiemann et al. 2003). Whether the TGF-β1 signaling cascade is affected in FaO cells after SPARC knock-down is currently investigated. In fact, recently we have published that *Snai1* induction in both FaO and MH cells requires the activation of TβRI (TGF-β receptor I) and NF-κB (Franco et al. 2010). Accordingly, Brancl and collaborators have demonstrated that the TGFβ–IKKα–Smad signaling pathway induces transcription of the genes encoding *Snai1* and *Snai2* (Brandl et al. 2010).

The role of SPARC in the suppressing functions of TGF-β in FaO cells is shown in Figure 15. Indeed, we describe here that SPARC depletion induced cell cycle arrest in G0/G1 phase and render cells more sensitive to the proapoptotic effects of TGF- β. As shown in Figure 15, the TGF-β treatment induces a significant increase of mitochondrial apoptotic death in SPARC-depleted cells compared with wild-type FaO cells. This novel function for SPARC suggests that its expression might be important for the TGF-β-driven EMT maintaining the cell survival and thus, facilitating the invasive process.

The role of SPARC in cell survival was originally described by Sage and colleagues (Sage et al. 1986) and identified as a stress response gene. Consistently, it has been recently reported that SPARC protects lens epithelial cells and pulmonary fibroblast from stress-induced apoptosis (Weaver et al. 2008, Chang et al. 2010). Importantly, Fenouille and colleagues demonstrate that in wild-type p53 melanoma cells SPARC can promote cell survival under basal and in response to stress through phosphorylation of Akt/MDM2

and thus, facilitating the p53 degradation (Fenouille et al. 2011). The involvement of this pathway is currently investigated in our FaO cell system.

Together, our work points to a role of Snai1 and SPARC in overcoming TGF-β tumor-suppressor effects in hepatocytes, hepatoma cells and in liver cancer cells, switching the response from tumor suppression to tumor progression, making them resistant to cell death and promoting them to undergo EMT and acquire invasive properties. In summary, the study of the molecules involved in EMT which confer resistance to cell death in pathogenic and non-pathogenic circumstances could help to better understand the mechanisms by which invasiveness is carried out in carcinogenic processes.

2. Tumorigenesis of *in vitro* TGF-β-chronically-treated tumor liver cells. Morphological and phenotypical analysis and apoptotic response.

2.1. Study of the metastatic potential of TGF-β-chronically-treated-FaO hepatoma cells by subcutaneous and intrasplenic implantation.

Previous results from our group showed that treatment of FaO rat hepatoma cells with TGF-β selects cells that survive to its apoptotic effect and undergo epithelial–mesenchymal transitions (EMT). After chronic treatment with TGF-β, we established a new cell line (TβT-FaO, from TGF-β-treated FaO, see Figure 17), which shows a mesenchymal, de-differentiated, phenotype in the presence of TGF- β and is refractory to its suppressor effects. TβT-FaO cells show higher capacity to migrate than that observed in the parental FaO cells (Bertran et al. 2009). We decided to inoculate both FaO and TβT-FaO cells subcutaneously and intrasplenically into Balb/c nude mice in order to explore its tumorigenic potential.

The subcutaneous injection of FaO and TβT-FaO cells in the flank of Balb/c nude mice allowed us to monitor and analyze the appearance of tumors in the two experimental groups. Tumor lesions appeared in both mice groups and there were no significant differences in the latency either in the tumor doubling time (Figure 18). Despite the fact that we couldn't find differences in the subcutaneous approach, there is a reduction in the proliferation rate (ki-67 immunohistochemistry), a high staining for p-Smad2 (Figure 20) and a slight delay in the maximum incidence of the tumours (Table I) in the TβT-FaO intrasplenic-induced tumors when compared to FaO-induced ones, suggesting that *in vitro* TGF-β treatment had affected somehow the tumor cell cycle progression.

The tumors obtained from FaO and TβT-FaO tumor injection were histological and macroscopically different. FaO-induced lesion (Figure 18) are consistent with the histology and morphology of hepatocellular carcinoma (HCC), which had been described as an area of mildly atypical tumor cells with abundant eosinophilic cytoplasm and little stroma and part of the tumor cells proliferates trying to replace the surrounding normal liver cell cords (Komuta et al. 2008). In contrast, the TβT-FaO-induced lesion shows dysplasic hepatocytes with irregular enlarged nuclei (higher nuclear-cytoplasmic ratio, N:C) and that can point out to stemness and more aggressive behavior in TβT-FaO tumors (Figure 18). It has been shown that stem cells or stem-like cells show a large N:C ratio (Zhou et al. 2004b) and the N:C ratio has a diagnostic and prognosis value. Anaplastic cells have abnormal large nuclei and increased N:C ratio owing to less-differenciated state. Additionally, aggressive tumors have high N:C ratio and poor survival (Carvalho et al. 1997). Malehmir and collaborators showed that the decrease in the N:C ratio in their treatment of thyroid carcinoma could be due to re-differenciation of their cells and these data might imply a decrease in the aggressive behavior of the tumor (Malehmir et al. 2012).

The intrasplenic approach revealed no significant differences between the percentages of metastasis in the two groups (Table I), but we could find differences in the morphology and phenotypic characteristics of the lesions. The liver tumors obtained from intrasplenic-FaO-inoculation showed a multifocal HCC with multiple clusters surrounded by connective tissue in all the tumor area (Figure 19). The liver tumor derived from intrasplenic injection of TβT-FaO cells displayed heterogeneous lesions mostly unifocal foci, bigger than FaO ones. Most of the lesions were more irrigated and seem less differentiated, reminding hepatoblastoma-like (HB) tumors (TβT-R lesions). However, white lesions also appeared, reminding hepatocellular-(HCC) or cholangiocarcinoma-like (CC) tumors (TβT-W lesions) (Figure 19). Small tumor areas of papillary and/or clear glandular formation with mucin production and abundant fibrous stroma are considered as typical features of CC (Komuta et al. 2008). The moderately differentiated tumors show distorted glandular or tubular patterns with formation and/or a cord-like pattern, while the poorly differentiated ones show markedly distorted tubular structures, with cellular pleomorphism (Nakanuma et al. 2003). The TβT-W lesion displayed multiple nodular areas collectively with cells polarized in circle, resembling enlarged ductal structures that pursue a cordlike pattern (Figure 19). The tumor capsule of hepatoblasts usually contains abundant blood vessels with some central necrotic areas that appeared as hemorrhagic holes filled with blood (Benesch et al. 1998). Interestingly some of the TβT-FaO-injected mice showed severe anemia. As we can see the TβT-R lesion doesn't present any organized structured like the other two types of tumors (Figure 19), but a large amount of sinusoids like a chemical induced hepatoblastoma in mice (Sakairi et al. 2001a).

The evaluation of cell proliferation ratio through ki67 immunostaining showed a higher proliferation rate in FaO-induced lesions when compared to TβT-W and TβT-R.

Interestingly the TβT-W liver tumor proliferates more than the TβT-R liver tumor (Figure 20). These results correlate with the growing rate observed in the corresponding tumor. Cells derived (FaO-D, TβT-W-D and Tβ-R-D) by means of Crystal Violet staining (Figure 23) and are in agreement with the characterization of TβT-R as a hepatoblastoma-like tumor, since it has been described that hepatoblastomas show weakly proliferating cell clusters despite their poorly differentiation state (Zimmermann 2005). We also assessed tumors for late apoptosis by using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Figure 20). Interestingly, the TβT-FaO-derived tumors did not show relevant appearance of apoptotic cells and the only tumor-derived cells that induced Caspase-3 activity in culture was FaO-D, the one corresponding to the FaO lesion (Figure 24). We previously demonstrated that cells that respond to TGF-β undergoing EMT are refractory to apoptotic stimuli (Valdes et al. 2002) and this phenomena is also observed in the TβT-FaO cells (Bertran et al. 2009). Our results indicate that the tumors formed from the TβT-FaO cells maintain certain resistance to apoptosis, which explains the similar growth to the FaO-derived tumors, although Ki67 staining is lower. TUNEL staining mostly correlated with ki67 positive cells in the FaO lesions, indicating that a compensatory hepatocyte proliferation might occur.

Next we analyzed the status of phosphorylated Smad2 (hallmark of TGF-β pathway activation) in the different lesions, detecting weak staining in FaO lesions when compared with TβT-W and mostly with TβT-R which was the strongest positive (Figure 20). Wu and collaborators found that p-Smad2 was strongly expressed in the nucleus and cytoplasm of non-tumor liver tissues in early stages of HCC. However, p-Smad2 was only located in the nucleus at late stages, implying that accumulation of p-Smad2 in the nucleus was closely correlated with liver pathological type and progression of hepatocarcinogenesis and may play an important role in HCC development (Wu et al. 2007). Another study showed that p-Smad2 was accumulated in the nucleus of hepatoma cell which might be correlated with hepatocellular tumorigenesis and development (Hua et al. 2005). Resistance to apoptosis and higher activation of the TGF-β pathway might indicate that TβT-FaO-derived lesions represent an advanced stage of liver tumorigenesis.

Regarding to the EMT hallmarkers, FaO lesions showed regions with strong and well-organized E-cadherin but, simultaneously, some regions appeared where cells have lost E-cadherin staining and have weak staining for Vimentin (Figure 21). This result suggests that EMT might occur *in vivo*. The literature shows a significant correlation between reduced expression of E-cadherin, the tumor grade and intrahepatic metastatis of HCC (Asayama et al. 2002). Other groups also found a correlation between loss in E-cadherin levels and metastasis in human HCC (Fransvea et al. 2008, van Zijl et al. 2009). Reduced expression of E-cadherin used to be accompanied by (partial) nuclear translocation of β-catenin, and significantly correlated with intrahepatic metastasis and poor survival of patients (Zhai et al. 2008). Interestingly,

Giannelli and collaborators have found an increase in the expression of the extracellular matrix (ECM) protein laminin (Ln)-5 with a concomitant downregulation of E-cadherin and dissociation of β-catenin from cell borders particularly at the tumor invasive front which correlated also with Snail and Slug (*Snai2*) expression in HCC (Giannelli et al. 2005), which reinforces the idea of EMT occurring in vivo during liver tumorigenesis.

The analysis of the TβT-FaO derived tumors revealed that the TβT-W lesions showed E-cadherin expression well organized and with a strong signal in the ductal, cholangiocyte-like, structures, although they conserved a middle intensity Vimentin expression in other areas (Figure 21). These changes in the adhesion molecules staining compared to its parental TβT-FaO cells could indicate that, after undergoing EMT *in vitro*, cells have suffered a transdifferentiation process *in vivo,* reminding a mesenchymal to epithelial (MET) process that allow them to acquire a cholangiocyte-like phenotype, although they come from an hepatoma in the original cell line. The TβT-R tumor displayed a weak staining for E-cadherin and a strong expression of Vimentin (Figure 21), which was corroborated in the phenotypic characterization of the TβT-W-D culture cells (Figures 25,27). The TβT-R liver lesion showed characteristics of an hepatoblastoma, which uses to lack E-cadherin expression (Anna et al. 2003, von Schweinitz et al. 1996), due to mutations in the β-catenin gene (Takayasu et al. 2001, Park et al. 2001, Anna et al. 2000). We haven't still checked β-catenin levels in these lesions, but here we show that these tumors present high levels of *Snai1*, *Snai2* (slug), Vimentin and SPARC (see Figures 22,27), characteristic of an EMT process.

In summary, the tumors derived from TβT-FaO cells may either conserve the mesenchymal-dedifferentiated phenotype, in whose case a hepatoblastoma might be formed, or transdifferentiate to other type of liver tumors, such is the case of the TβT-W cholangiocarcinoma-like lesions. One of the most relevant conclusions after the phenotypic analysis of the tumors derived from FaO and TβT-FaO cells is that EMT can occur in vivo, since FaO lesions show loss of E-cadherin and expression of Vimentin, but also MET can occur, since the lesions induced with the TβT-FaO cells (mesenchymal phenotype) show recovery of E-cadherin expression and epithelial features. This reflects the high plasticity of liver tumor cells. We have previously shown that TGF-β-induced EMT confers to the hepatocytes and liver tumor cells characteristics of liver progenitors (del Castillo et al. 2008, Caja et al. 2011a). In agreement with this idea, TβT-FaO cells appear to become as progenitor liver cells, since in vivo MET allows them to transdifferentiate to a cholangyocitic-like cell. It is well known that EMT also confers to breast cancer cells a CD44+/CD24- cell surface profile that is proposed as cancer stem cell (Al-Hajj et al. 2003, Liu et al. 2007a, Honeth et al. 2008). TGF-β1 and SPARC are overexpressed in this CD44+/CD24- population and they have proved that Slug overexpression generate cells with enhanced mammosphere forming ability (Bhat-Nakshatri et al. 2010).

Connecting with this idea, FaO tumors mostly express Cytokeratin 18 as the parental FaO cell line does (Bertran et al. 2009) but not Cytokeratin 7 nor Cytokeratin 19, which

are typical of de-differentiated hepatocytes and differentiated cholangiocytes. In contrast, TβT-FaO derived tumors express the three CK18, CK7 and CK19, with enrichment on CK7/19 over the CK18 in the case of the TβT-W lesions (Figure 22). In support of these results Zulehner and collaborators have recently reported that intrasplenic liver tumors generated by hepatocytes overexpressing oncogenic v-Ha-Ras expressed CK8/CK18 but not those tumors generated from the same hepatocytes treated with TGF-β for 14 days and which have undergone EMT. These last lesions failed to express CK8 and CK18 and displayed strong staining for CK19, suggesting a more immature phenotype because the cells that have undergone EMT lack hepatocytic differentiation (Zulehner et al. 2010). In HCC, a substantial number (ranging from 28 to 50%) of patients express markers of progenitor/biliary cells such as CK7, CK19 and OV6 suggesting a progenitor cell origin (Roskams 2006). Fan and collaborators postulated that circulating cancer stem cells are likely the source of HCC recurrence and might be an additional prognosis indicator of early tumor vascular invasion and their elimination should be a reasonable approach (Fan et al. 2011). Furthermore, CK19 was correlated with vascular invasion in HCC (Ding et al. 2004) and with an important risk factor for ETR (early tumor recurrence), regardless of the presence or absence of p53 or β-catenin mutation (Yuan et al. 2011). HCCs positive for CK19 were found to have CC-like differentiation associated with poorer differentiation, higher level of cellular proliferation, and poorer survival (Andersen et al. 2010, Uenishi et al. 2003, Wu et al. 1999, Yoon et al. 1999). The fact that cholangiocarcinomas contain both hepatocytic and cholangiocytic features is also important evidence to support the hypothesis that hepatic malignancy could be stem cell. Zhang and collaborators (Zhang et al. 2008) found positive staining of both CK7 and CK19 in the glandular areas (as other groups, (Komuta et al. 2008)) whereas hepPar1 (hepatocyte marker (Evarts et al. 1989)) was predominantly expressed in the hepatocytic areas. The tumor cells of the transition areas showed simultaneously cytoplasm expression of HepPar 1 and CK19; as we also found hepatocytic (CK18) and biliary markers (CK19, CK7) in the TβT-W and TβT-R lesions (Figures 22,26,27). Interestingly, hepatocytes expressing constitutive Wnt/β-catenin generated tumors showing duct-like cell structures similar to the morphology exhibited in cholangiocarcinoma (see the corresponding ductal structures in TβT-W tissue and TβT-W-D cell culture in Figures 19,23,28). These data suggest that malignant hepatocytes induced by TGF-β or expressing constitutive Wnt/β-catenin might have the potential to dedifferenciate into hepatic progenitors (Zulehner et al. 2010). Comparative functional genomics reveal that the CK19 associated gene expression signature predict the patient survival and tumor recurrence (Andersen et al. 2010). Lee and collaborators (Lee et al. 2006c) determined that the gene expression profile of hepatoblastoma subtype and fetal rat hepatoblasts were closely related suggesting than the CK19 positive foci found in HCC by the same group (Andersen et al. 2010) might be of hepatic progenitor origin.

2.3. Isolation and characterization of intrasplenic-tumor-obtained cell lines.

To analyze the relevance of the results obtained in the immunostaining studies performed in the liver tumours and to better characterize the resulting phenotype, we decided to obtain primary cultures from the different lesions.

As it's showed in Figure 23, morphology of the FaO-D cells in culture is similar to the parental FaO and also regarding F-actin filaments (Figure 25 and (Bertran et al. 2009)). Both cultures showed cells in a parenchymal structure and displaying typical epithelial actin distribution. Respect to the TβT-FaO derived cultures, TβT-R-D cells reminded the TβT-FaO cells used to originate the tumors, with spindle-like and fibroblastoid apperance cells that organize their actin cytoskeleton into stress fibers (Figures 23,25 and (Bertran et al. 2009)). However, the TβT-W-D culture has a heterogeneous phenotype with many cells organized in parenchymas showing pericellular F-actin, but also other cells that do not display an epithelial phenotype (Figure 25). Guillouzo and collaborators (Guillouzo et al. 2007) described a new hepatoma cell line (HepaRG) derived from a human hepatocellular carcinoma which exhibited unique features. When cells are seeded at low density adquired elongated undifferenciated morphology, actively divided and after having reached confluency formed typical hepatocyte-like colonies surrounded by biliary epithelial-like cells that showed canaliculus-like structures. The TβT-W-D cells (Figure 23) phenotype recapitulates the Guillouzo's findings, which they relate to characteristics of hepatoblast/liver stem cells in HepaRG cells.

Considering these results and the previous ones obtained by our group that related the EMT induced by TGF-β with the acquisition of a liver stem cell phenotype (Bertran et al. 2009), we decided to explore the differentiation state of all this obtained cell cultures compared to the parental cell lines. Hepatocyte nuclear factor (HNF)-4 is a transcription factor essential for hepatocyte differentiation (Pontoglio et al. 1996, Li et al. 2000, Hayhurst et al. 2008). It has been proposed (Limaye et al. 2010) that gradually loss in the expression of HNF4α, up-regulation of HNF1β expression and appearance of Cytokeratin 19 are characteristic of a differentiation process to a bile duct lineage. HNF4 shows an inverse expression pattern with CK19 staining in HCC (Andersen et al. 2010) and has been implicated in the EMT of slow-growing mouse HCC cells to highly invasive, fast-growing and dedifferentiated cells. Restoration of HNF-4α expression in fast-growing HCC cells reversed the progressive phenotype by re-establishing hepatocytes with intact epithelial organization and reduced proliferation, indicating that loss of HNF-4 is an important determinant of HCC progression (Lazarevich et al. 2004). Taken together this finding we can observe in Figure 27 that the TβT-FaO, TβT-W-D and TβT-R-D cultures have lost the expression of HNF4α without affecting HNF3β (which is expressed in earlier stages of liver differentiation) suggesting a transdifferentiation process has occurred due to TGF-β chronic treatment and that cells

compromised with. Furthermore, as observed in the tissue immunohystochemistry, TβT-FaO derived cell cultures express CK19, which reinforce the hypothesis of a step-back to an earlier stage of differentiation, which might allow cells to later differentiate to both hepatocytes and cholangiocytes. Furthermore, these "in vitro" results corroborate the previous hypothesis formulated from the "in vivo" results that chronic treatment with TGF-β confers to FaO hepatoma cells to form tumors with a less differentiated phenotype.

Interestingly, EpCAM (Epithelial Cell Adhesion Molecule) a marker found to be expressed in normal cholangiocytes and hepatoblasts (Tanaka et al. 2009, Okabe et al. 2009b) was found only in FaO-TβT, TβT-W-D and TβT-R-D (Figure 27). It's also been reported that colonies of biliary tree stem/progenitors uniformly expressed EpCAM and also variable amount of markers for mature hepatocytes and cholangiocytes when cultured on plastic (Cardinale et al. 2011). The kit receptor was also found to be a stem cell marker, but it is also highly expressed in cholangiocytes (Crosby et al. 2001). Interestingly, we found it highly expressed in TβT-W-D cultures (Figure 27,28). Expression of c-kit has also been considered to have important prognosis implications (Ernst et al. 1998, Tonary et al. 2000). Zhang and collaborators (Zhang et al. 2008) found that 83.3% of CHC cases expressed c-kit, which was noted mainly in small cells within the transition zone that showed intermediate features; of these, 70% showed simultaneously expression of hepatocytic and/or cholangiocytic markers. Sakairi and collaborators (Sakairi et al. 2001a) found a correlation with overexpression of c-kit and high proliferation rate in hepatoblastoma, although contribution of c-kit to genesis and progression of hepatoblastoma is still uncertain. Interestingly, TβT-R-D (with no expression of c-kit in Figure 28) shows a lower proliferation rate when compared to TβT-W-D (crystal violet staining in Figure 23 and ki67 staining in Figure 20). Hepatic progenitor cells (Yin et al. 2002, Fougere-Deschatrette et al. 2006) are capable of forming duct-like structures when placed on a matrigel substract and liver progenitor cells developed cholangiocyte-type epithelial polarity in three –dimensional culture (Tanimizu et al. 2007). The only culture that was capable of forming ductular structures on matrigel were the TβT-W-D (Figure 28), confirming its cholangiocytic lineage.

It is interesting to point out that our results suggest the enormous plasticity of the liver cells lineage, since the only incubation with TGF-β might confer to theorically hepatocytes-like cells the capacity to transdifferentiate to cholangiocytes. In agreement with these results, the group of Michalopoulos have shown that rat hepatocytes had the potential to differentiate into bile duct-like cells in vitro (Michalopoulos et al. 2001, Michalopoulos et al. 2002, Michalopoulos et al. 2005, Nishikawa et al. 2005, Limaye et al. 2008), after incubation with epidermal growth factor (EGF) and/or hepatocyte growth factor (HGF), which became as potent inducers of biliary phenotype. Transforming growth factor-β1 (TGF-β1) and basic fibroblast growth factor (FGF-b) are also well-known growth factors that have key roles in the development and differentiation of the liver (Zaret 2001, Lemaigre and Zaret 2004,

Yoshida et al. 2007, del Castillo et al. 2008). It is well known the implication of TGF-β/BMP and Notch signaling pathways in ductular differenciation of fetal murine hepatoblasts and they are found to be activated in response to Matrigel (Ader et al. 2006).

In summary, the intraesplenic inoculation of FaO rat hepatoma cells gave rise to tumors that resemble HCC while chronic *in vitro* TGF- β treatment of FaO cells changed their tumorigenic potential. Tumor growth was similar, but phenotype of lesions reflected a stem-like phenotype which provokes the appearance of less differentiated tumors (hepatoblastomas) or transdifferentiation to a different liver tumor lineage (cholangiocarcinoma). The heterogeneity may result from tumor cell renewal and adaptation to specific microenvironment. The presence of cancer stem cells may be another contributor of the heterogeneity (Vermeulen et al. 2008, Bioulac-Sage et al. 2001). The results obtained here may also have implications in the histogenesis of combined hepatocellular cholangiocarcinomas and hepatoblastomas. Indeed, the component of cholangiocarcinoma might originate from the transdifferention of the HCC component, forming a combined hepatocellular cholangiocarcinoma (CHC) that exhibits focal or diffuse immunohistochemical expression of CK19, as has been previously suggested (Zhang et al. 2008). The histogenesis of mouse hepatoblastoma remains as a matter to be discussed further, and additional studies are necessary to clarify the origin (Sakairi et al. 2007). Considering all the available evidence and results here presented, we might propose that hepatoblastomas resemble some hepatic undifferentiated cells including de-differentiated hepatocytes and bipotent hepatic progenitor cells and it is worthy to note that they might also be generated through a TGF-β-mediated transdifferentiation process.

IX. CONCLUSIONS

First. Targeting knock-down of Snail1 in murine hepatocytes and human hepatocarcinoma cells increases TGF-β-induced mitochondrial-mediated apoptosis, correlating with inhibition in the expression of epidermal growth receptor ligands and dysregulation of the balance between pro and antiapoptotic members of the Bcl-2 family.

Second. Targeting knock-down of SPARC in hepatoma cells impairs Snail1 upregulation and increases TGF-β-induced-apoptosis.

Third. Chronic *in vitro* TGF-β treatment of FaO cells changed their tumorigenic potential. Tumor growth was similar, but phenotype of lesions reflected a stem-like phenotype.

Fourth. Primary culture of cells from tumors obtained after intrasplenic injection of TGF-β-treated hepatoma cells revealed the appearance of less differentiated tumors (hepatoblastomas) or transdifferentiation to a different liver tumor lineage (cholangiocarcinoma).

CONCLUDING REMARK

Tumorigenic cells can circumvent the tumor supressor effects of TGF-β by directing it towards the induction of Snail1 and SPARC expression, which promote both EMT and survival and might change its tumorigenic potential to provoke the appearance of less differentiated tumors or the transdifferentiation to a different liver tumor lineage.

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