# Reprogramming of B cells into macrophages: mechanistic insights.

Alessandro Di Tullio

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DIRECTOR

Thomas Graf

Gene Regulation, Stem Cells and Cancer Department.
Center for Genomic Regulation (CRG), Barcelona

UNIVERSITAT POMPEU FABRA

*Alle mie nipotine*

*To my nieces*

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## THESIS ABSTRACT

Our earlier work has shown that pre-B cells can be converted into macrophages by the transcription factor C/EBPα at very high frequencies and also that a clonal pre-B cell line with an inducible form of C/EBPα can be converted into macrophage-like cells. Using these systems we have performed a systematic analysis of the questions whether during transdifferentiation the cells retrodifferentiate to a precursor cell state and whether cell cycle is required for reprogramming.

As for the first question, a transcriptome analysis of transdifferentiating cells showed that most genes are continuously up or downregulated, acquiring a macrophage phenotype within 5 days. In addition, we observed the transient reactivation of a subset of immature myeloid markers, as well as low levels of the progenitor markers *Kit* and *Flt3* and a few lineage inappropriate genes. Importantly, we were unable to observe the re-expression of cell surface marker combinations that characterize hematopoietic stem and progenitor cells (HSPCs), including c-Kit and Flt3. This was the case even when C/EBPα was activated in pre-B cells under culture conditions that favor HSPC growth or when the transcription factor was activated in a time limited fashion.

As for the second question, using the C11-inducible pre-B cell line, time-lapse experiments showed that a subpopulation of about 8% of the pre-B cells did not divide before acquiring macrophage properties, with the majority of cells dividing once and a few percent dividing twice. In agreement with these results we found that 8% of the induced cells did not incorporate BrdU during reprogramming. Importantly, the non-dividing cell subset expressed the highest levels of C/EBPα and was the fastest in acquiring a macrophage phenotype. Inhibition of DNA synthesis by aphidicolin led to an impairment of transdifferentiation in >70% of the cells, suggesting a requirement for traversing the cell cycle. However, sorting pre-B

cells into G0/G1 and G2/M fractions followed by induction showed no significant differences in the reprogramming kinetics. Finally, we showed that knocking down p53 in the inducible pre-B cells does not alter their conversion into macrophages, suggesting that an acceleration of the cell cycle has no effect.

Together, our findings show that the conversion of pre-B cells to macrophages does not involve overt retrodifferentiation and that high concentrations of C/EBPα bypass the cell cycle-dependency of immune cell transdifferentiation.

## RESUMEN DE TESIS

Recientemente, nuestro grupo ha demostrado que las células pre-B se pueden reprogramar a macrófagos mediante la sobreexpresión del factor de transcripción C/EBPα, con una eficiencia elevada. Así mismo, mediante la expresión de la forma inducible de C/EBPα en una línea de células pre-B (C11), éstas también se puede convertir en células similares a macrófagos. Usando este sistema hemos estudiado si durante el proceso de trans-diferenciacion las células requieren volver a un estadio de célula precursora, y si el ciclo celular es necesario para este proceso.

En cuanto a la primera cuestión, el análisis del transcriptoma de células trans-diferenciadas mostró que la expresión de la mayoría de los genes están regulados durante todo el proceso bien aumentando o disminuyendo, y que adquieren el fenotipo de macrófago a los 5 días después de iniciar el proceso. Así mismo, se observó la reactivación transitoria de un grupo de genes que codifican para marcadores de células mieloides inmaduras; también cabe destacar que observamos una disminución en la expresión de los genes expresados en células progenitoras Kit y Flt3, así como de genes de linajes impropios. Es importante destacar que nunca hemos llegado a observar la expresión de combinaciones de marcadores de superficie característicos de las células madre hematopoyéticas y las células progenitoras (HSPCs), incluyendo c-Kit y Flt3, mediante el análisis por citometría de flujo. Estos resultados se reprodujeron incluso cuando C/EBPα se sobreexpresó en células pre-B que fueron cultivadas en condiciones que favorecen el crecimiento de las HSPC o cuando el factor de transcripción se activó de forma limitada en el tiempo.

En cuanto a la segunda pregunta, usando la línea de células inducibles pre-B C11, el análisis mediante microscopia a diferentes tiempos después de la inducción de la reprogramación mostraron que una subpoblación de aproximadamente el 8% de las células pre-B no se dividen antes de adquirir

las propiedades de macrófago, mientras que la mayoría de las células se dividen sólo una vez y un pequeño porcentaje dos veces antes de que se reprogramen totalmente a macrófagos. De acuerdo con estos resultados se encontró que un 8% de las células inducidas no incorporan BrdU durante la reprogramación. Es importante destacar que el subconjunto de células que no se dividen expresan los niveles más altos de C/EBPα, con lo que cabe pensar que la adquisición del fenotipo de macrófago es más rápida en estas células.

La inhibición de la síntesis de ADN por afidicolina bloqueó la transdiferenciación en mas de un 70% de las células, lo que sugiere que la correcta progresión del ciclo celular es un requisito para la transdiferenciación. Sin embargo, al separar la linea de células pre-B C11 en fracciones G0/G1 y G2/M seguido de la inducción, la cinética de la reprogramación no mostró diferencias significativas. Por último, también demostramos que la reducción en la expresión de p53 en las células pre-B inducibles no altera el proceso de conversión a macrófago, lo que sugiere que la aceleración del ciclo celular no tiene ningún efecto.

En conjunto, nuestros resultados muestran que la conversión de células pre-B a macrófagos no requiere retro-diferenciación y que las células con una expresión mayor de C/EBPα pueden llegar a prescindir de la dependencia del ciclo celular para la trans-diferenciación de las células inmunitarias.

## PREFACE

Classic experiments such as somatic cell nuclear transfer into oocytes or cell fusion demonstrated that differentiated cells are not irreversibly committed to their fate. More recent work has built on these findings leading to the discovery of defined factors that directly induce the conversion of one cell type into another, including between distantly related cells from different germ layers. These examples of cell transdifferentiation raise the possibility that any cell type may be converted into any other if the correct combinations of reprogramming factors are known. Our reprogramming system of pre-B cells into macrophages provides a perfect tool for understanding the mechanisms underlying cell transdifferentiation, which may be useful for both basic biology and regenerative medicine.

# PART I

# INTRODUCTION AND AIMS

# Introduction

## 1. History and strategies of reprogramming

In multicellular organisms cells are functionally heterogeneous because of the differential expression of genes. Historically, this difference had been thought to involve the retention of those genes that were expressed in a particular tissue and the elimination of those that were silenced. Cloning experiments in mammals and amphibians refuted this idea (Gurdon and Byrne, 2003). They clearly demonstrated that differential gene expression is the result of reversible epigenetic changes that are gradually imposed on the genome during development. In addition it was shown by cloning that differentiated mammalian cells are genetically identical to early embryonic cells (Wakayama et al., 1998; Wilmut et al., 1997). Mammalian development is a unidirectional process, which begins with the formation of a unicellular zygote, continues with the growth of stem cells and ends with the establishment of the 220 specialized cell types of the body.

Stem cells, which are characterized by the ability to both self-renew and to generate differentiated functional cell types, can be classified according to their developmental potential (Table 1).

| **Potency** | **Sum of developmental options accessible to cell** |
|---|---|
| Totipotent | Ability to form all lineages of organism; in mammals only the zygote and the first cleavage blastomeres are totipotent |
| Pluripotent | Ability to form all lineages of body. Example: embryonic stem cells |
| Multipotent | Ability of adult stem cells to form multiple cell types of one lineage. Example: hematopoietic stem cells |
| Unipotent | Cells form one cell type. Example: spermatogonial stem cells (can only generate sperm) |
| Reprogramming | Increase in potency, dedifferentiation. Can be induced by nuclear transfer, cell fusion, genetic manipulation |
| Lineage reprogramming, Transdifferentiation | Notion that somatic stem cells have broadened potency and can generate cells of other lineages |

Table 1. Definition of some terms. Adapted from (Jaenisch and Young, 2008).

In mammals only the zygote and early blastomeres are totipotent and can generate the whole organism including extra embryonic tissues and germ cells. Mouse embryonic stem (ES) cells are an example of pluripotent cells that can self-renew and generate all cell types of the body in vivo and in culture but are not able to generate the extra embryonic trophoblast lineage (Rossant, 2008). Multipotent cells such as hematopoietic stem cells can give rise to all cell types within one particular lineage (Orkin and Zon, 2008). Spermatogonial stem cells are an example of unipotent stem cells, as they can only form sperm (Cinalli et al., 2008).

Differentiated adult cells generally do not switch fates; for example, neurons do not spontaneously become hepatocytes. Nevertheless, several classic studies suggested a "plasticity" of "committed" cells of the embryo, since their fate can be modified when they are explanted and exposed to a different microenvironment (Gehring, 1967; Hadorn, 1966; Le Lievre and Le Douarin, 1975). Several conceptual and technological breakthroughs have been instrumental for the discovery of this plasticity and transcription factor induced cell reprogramming (Fig. 1).

In one of these studies, cells from the imaginal discs of Drosophila melanogaster pupae were serially transplanted into the abdomen of an adult fly, and "transdetermination" was observed: cells that were originally destined to form genital structures gave rise to leg or head structures and, eventually, on subsequent transplantations, to wings (Gehring, 1967; Hadorn, 1966). Moreover, the process was directional in that, to "transdetermine" genital disks into wings they first had to go through a stage fated either to a leg or an antenna (Ursprung and Hadorn, 1962). Even though the frequency of such switches in cell fate was low, these experiments provided evidence that explanted cells were surprisingly plastic.

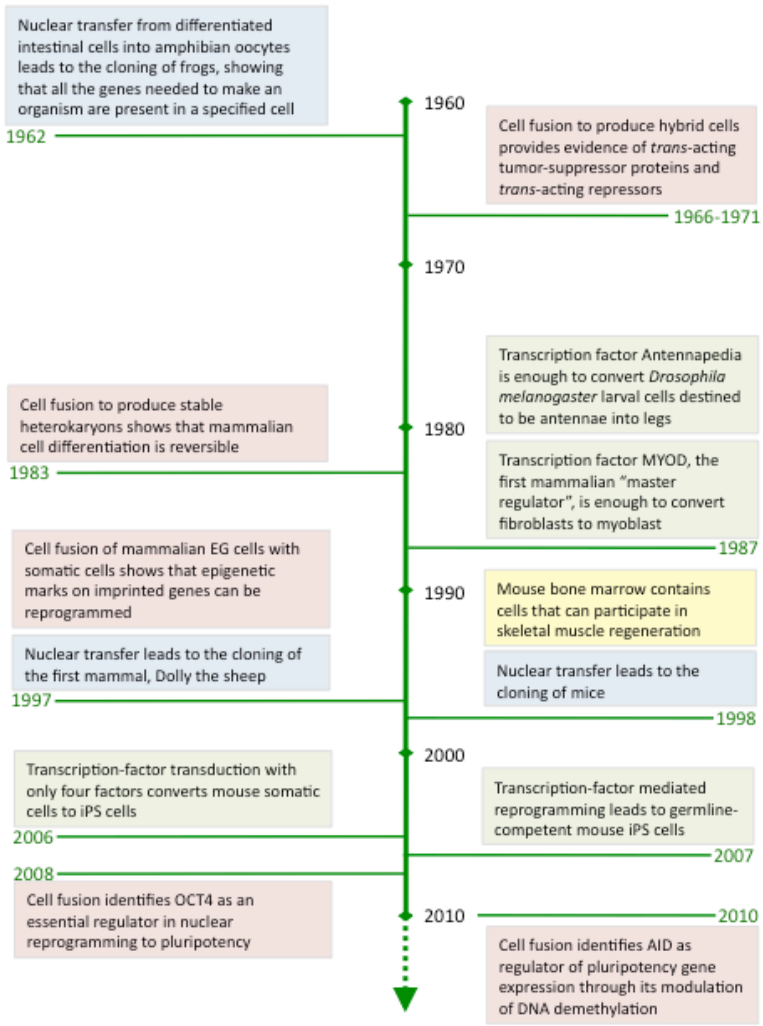


Figure 1 | Timeline of discoveries in nuclear reprogramming. Four approaches to nuclear reprogramming are described: somatic cell nuclear transfer (blue), cell fusion (pink), culture-induced reprogramming (yellow) and reprogramming by defined transcription factors (green). These complementary approaches have provided synergistic insights for almost 50 years and continue to inform the understanding of nuclear reprogramming and influence medical advances. EG cell, embryonic germ cell. Modified from (Yamanaka and Blau).

Cell plasticity was also found in another study from Le Lievre and Le Douarin in which they transplanted cells from quails to chickens (Le Lievre and Le Douarin, 1975): these cells were histologically distinct, enabling them

to be tracked, but were sufficiently similar to be able to participate in normal development on transplantation. They showed that explanted neural crest cells adopt new fates (bone, cartilage and connective tissue) that are not dictated by their original location in the avian embryo but by their new cellular neighbourhood.

It might seem akward that a specialized cell would maintain the potential to reactivate genes typical of another cell type, given the possibility that genes could be inappropriately activated. Yet, it has been conclusively shown that cell fate can be reversed in a defined specialized cell type, returning the cell to an embryonic state, using four different nuclear reprogramming approaches: somatic nuclear transfer, cell fusion, culture-induced reprogramming and reprogramming by defined transcription factors (Fig. 2).

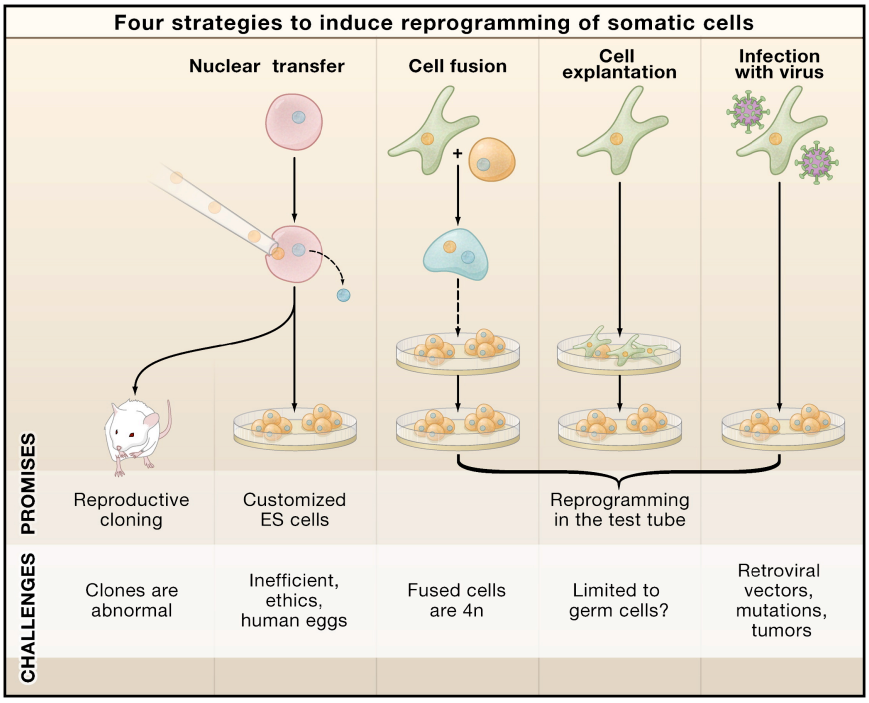


Figure 2. Four Strategies to Induce Reprogramming of Somatic Cells (1) Nuclear transfer involves the injection of a somatic nucleus into an enucleated oocyte, which, upon transfer into a surrogate mother, can give rise to a clone ("reproductive cloning"), or, upon explanation in culture, can give rise to genetically matched embryonic stem (ES) cells

("somatic cell nuclear transfer," SCNT). (2) Cell fusion of somatic cells with ES cells results in the generation of hybrids that show all features of pluripotent ES cells. (3) Explantation of somatic cells in culture selects for immortal cell lines that may be pluripotent or multipotent. At present, spermatogonial stem cells are the only source of pluripotent cells that can be derived from postnatal animals. (4) Transduction of somatic cells with defined factors can initiate reprogramming to a pluripotent state. Adapted from (Jaenisch and Young, 2008).

These four experimental models show that, with few exceptions (such as homologous recombination in lymphocytes), specialized somatic cells retain the genetic information that is needed for them to revert to ES cells and that the genes of the somatic cells have not been permanently inactivated. In addition, they provide evidence that cellular "memory" is dynamically controlled and subject to changes induced by perturbations in the stoichiometry of the transcriptional regulators present in the cell at any given time.

It has also been conclusively shown, starting with the work of Weintraub in 1987 (Davis et al., 1987), that a somatic cell type can be converted into another specialized cell type, using overexpression or ablation of cell specific transcription factors (lineage reprogramming or transdifferentiation) (Graf and Enver, 2009).

These findings have led to great excitement regarding the potential of these cells for improving the understanding and treatment of disease and have highlighted the need for a better mechanistic understanding of the reprogramming process.

## 2. Reprogramming of somatic cells into embryonic stem cells

ES cells are derived from the inner cell mass of blastocyst stage embryos and have the unique capacity to proliferate extensively while maintaining pluripotency. Since theoretically they have the capacity to develop into any cell type, the generation of ES cell lines from human blastocyst embryos (Thomson et al., 1998) has offered the possibility of using these cells as a donor source for cell transplantation therapies. Potential clinical applications include treatment of degenerative diseases such as juvenile diabetes, Parkinson's disease and heart failure as well as spinal cord injury and burns. However, as is the case for organ transplantation, tissue rejection is a concern for ES cell transplantation. One possible means to avoid immune rejection is the reprogramming of the nuclei of differentiated cells to an ES cell-like, pluripotent state, and using these cells to generate appropriate donor cells for transplantation. This process is of interest for three reasons. First, identifying how reprogramming takes place can help us understand how cell differentiation and specialized gene expression are normally maintained. Second, nuclear reprogramming represents a first major step in cell-replacement therapy, in which defective cells are replaced by normal cells of the same or a related kind but derived from a different cell type. Eventually, it may be possible to derive replacement heart, pancreas, or other types of cells from the skin of the same individual, thereby avoiding the need for immunosuppression. Third, nuclear reprogramming enables the culture of lines of cells from diseased tissues, and hence permits to analyze the nature of the disease and to screen for therapeutic drugs.

It is important to realize, however, that in contrast to cells growing in the embryo, cells in tissue culture are exposed to different selective conditions, and this may result in cell states that are unlike those seen in vivo. Consequently, concepts such as pluripotency, multipotency, or differentiation of cultured cells rely on operational criteria and are typically

assessed by different functional and molecular standards. The least stringent functional assay for the developmental potential of a cultured cell is in vitro differentiation followed, with increasing stringency, by the generation of teratomas (germ cell tumors), chimera formation, and germ line contribution. In the light of this, several different strategies have been employed to induce the conversion of differentiated cells into an embryonic state, such as somatic cell nuclear transfer, cell fusion, culture-induced reprogramming and reprogramming by defined trancription factors (Fig. 2).

## 2.1. Somatic cell nuclear transfer

Somatic cell nuclear transfer (SCNT) is a technique, which denotes the introduction of a nucleus from a donor somatic cell into an enucleated oocyte to generate a cloned animal such as Dolly the sheep (Wilmut et al., 1997). The generation of live animals by SCNT demonstrated that the stable epigenetic state of differentiated somatic cells is not irreversibly fixed but can be reprogrammed to an embryonic state capable of directing development of a new organism. However, because no genetic marker was available in the initial cloning experiments, it remained an open question whether terminally differentiated cells could be reprogrammed to a totipotent state. Different groups finally put to rest this argument dimostrating that terminal differentiation does not restrict the potential of the nucleus to support development, with the successful generation of cloned mice from genetically marked lymphoid cells (Hochedlinger and Jaenisch, 2002) or from postmitotic neurons (Eggan et al., 2004). However it has been suggested that the differentiation state of the donor cell affects the efficiency of producing cloned animals, with less differentiated cells being more amenable to epigenetic reprogramming (Eminli et al., 2009): the generation of cloned ES cells from neurons was less efficient than that from neural stem cells (Blelloch et al., 2006; Inoue et al., 2007) and also direct cloning of mice from

skin stem cells was more efficient than cloning from transiently amplifying keratinoyctes (Li et al., 2007). However it remains controversial whether cloning efficiency decreases with progressive cell differentiation in all cases, in that the cloning process is affected by many other parameters, such as cell cycle and the physical characteristics of the donor nucleus. For example, it has been argued that nuclei from granulocytes are more efficient donors than nuclei from hematopoietic stem cells (Sung et al., 2006).

Nuclear cloning is nevertheless an inefficient process due to imperfect reprogramming, which results in the death of most clones soon after implantation or birth of clones with serious abnormalities (Yang et al., 2007). The developmental defects in cloned animals might in part be due with the fidelity of genomic reprogramming (Simonsson and Gurdon, 2004), owing to a failure to erase completely the "epigenetic memory" of the cell. The frequency of abnormalities in cloned animals that have been generated by nuclear transfer suggests that a better understanding of the mechanisms of gene regulation, particularly those of epigenetic memory, is required. Moreover, it was postulated that cloning of mammals could be accomplished only when oocytes rather then fertilized eggs were used as nuclear recipients (McGrath and Solter, 1984). However, later it was shown that cloning is possible if oocytes satge xx are used (Wilmut, Wakayama). This result posed a significant impediment to the potential of nuclear transplantation approaches for therapeutic application in humans because of the difficulty in obtaining unfertilized human oocytes. Egli (Egli et al., 2007) and Greda et al (Greda et al., 2006) showed that cloned ES cells and mice can be generated from somatic donor nuclei transplanted into enucleated zygote recipients if drug-induced synchronization of donor cells and zygote is employed. This could be a good strategy if adapted to the human system and could solve major practical problems that hamper the eventual application of nuclear transplantation for medicine.

## 2.2. Cell fusion

Another way to demonstrate the epigenetic reprogramming of somatic nuclei to an undifferentiated state are murine hybrids produced by fusion of embryonic cells with somatic cells. Hybrids between various somatic cells and embryonic carcinoma cells (Solter, 2006), embryonic germ (EG) cells, or ES cells (Zwaka and Thomson, 2005) share many features with the parental embryonic cells, indicating that the pluripotent phenotype is dominant in such fusion products. Human ES cells have also the potential to reprogram somatic nuclei after fusion (Cowan et al., 2005). A crucial question raised by these experiments was whether the chromosomes of the somatic cell had been reprogrammed to pluripotency, or whether they were simply retained as silent cargo. At the molecular level, the expression of genes representative of all three germ layers in teratomas produced from hybrids (Tada et al., 2003), the demethylation and reactivation of genes essential for pluripotency (Cowan et al., 2005; Tada et al., 2001) and the reactivation of the silent X chromosome in female lymphocyte–ES-cell hybrids (Tada et al., 2001), suggested that the somatic chromosomes had undergone epigenetic reprogramming. Two key questions arising from fusion experiments are whether DNA replication is needed for reprogramming and whether the ES-cell nucleus or cytoplasm is required. The requirement for DNA replication for reprogramming is not very clear. Although one ES cell–somatic cell fusion experiment suggested that replication is essential for reprogramming (Do and Scholer, 2004), nuclear transfer experiments indicated the presence of a replication-independent mechanism, possibly involving an active DNA demethylase (Simonsson and Gurdon, 2004). The different results might be due to biological differences in the cell types (ES cell versus oocyte) and/or technical differences in the assays used (cell fusion versus nuclear transfer).
The second question was addressed by separating the nuclear compartment (karyoblast) from the cytoplasmic compartment (cytoblast) of an ES cell and

then fusing them individually with neuronal cells isolated from neurospheres (Do and Scholer, 2004). In hybrids produced with ES-cell karyoblasts, reactivation of an *Oct4*–green fluorescent protein transgene was detected in the fusion partner. By contrast, fusion of neurosphere cells with ES-cell cytoplasts gave no Oct4 protein signal, suggesting that nuclear factors are essential for molecular reprogramming. This conclusion is consistent with cloning experiments in amphibians (Byrne et al., 2003) and mice (Wakayama et al., 1998), which indicate that successful reprogramming depends on direct injection of nuclei into the germinal vesicle or into a metaphase oocyte, where nuclear factors are available in the cytoplasm.

Reprogramming of somatic cells to pluripotency is a potentially attractive approach to generate customized cells for therapy without having to rely on nuclear transfer (Cowan et al., 2005). However, for this approach to be viable, the ES-cell nucleus needs to be removed from the hybrid in order to generate diploid customized cells for transplantation therapy. If DNA replication and cell division are required for complete reprogramming it will be difficult, if not impossible, to selectively eliminate the entire set of ES-cell chromosomes from the hybrids.

### 2.3. Culture-induced reprogramming

The approaches discussed so far require the exposure of somatic nuclei to nuclear/cytoplasmic factors of an oocyte or ES cell to elicit nuclear reprogramming. An important issue has been whether pluripotent cells can be derived not only from the embryo but also from adults without previous manipulation of their nuclei. Several reports have described the derivation of multipotent or pluripotent cell lines from adult tissues, including multipotent adult progenitor cells (MAPCs) from adult bone marrow (Jiang et al., 2002) and unrestricted somatic stem cells (USSCs) from human newborn umbilical

cord blood (Kogler et al., 2004). These cells were shown to differentiate into cell types indicative of all three germ layers in culture and, when a single MAPC was injected into blastocysts, one extensive chimaera was reported (Jiang et al., 2002). Although these results are intriguing, they await confirmation by independent laboratories. Also, it remains to be seen whether MAPCs and USSCs can functionally contribute to somatic tissues in animal models of disease or injury.

Donor cells from the germ cell lineage such as primordial germ cells ("PGCs") or spermatogonial stem cells are known to be unipotent in vivo, but it has been shown that pluripotent ES-like cells (Kanatsu-Shinohara et al., 2004), or multipotent adult germ-line stem cells ("maGSCs") (Guan et al., 2006), can be isolated after prolonged in vitro culture. ES-like cells expressed all the markers of pluripotent cells, formed teratomas after transplantation and gave rise to chimaeric animals that transmitted to the germ line. Thus, these cells represent the only clear example of the derivation of pluripotent cells from a normal neonatal or adult mammal, and might be useful for studying genetic diseases in different cell lineages. Recently, multipotent adult spermatogonial stem cells ("MASCs") were derived from testicular spermatogonial stem cells of adult mice, and these cells had an expression profile different from that of ES cells (Seandel et al., 2007) but similar to epiblast stem cells ("EpiSCs"), which were derived from the epiblast of postimplantation mouse embryos (Brons et al., 2007). While both MASCs and EpiSCs were able to differentiate in vitro and to generate teratomas in vivo, they were unable to form chimeras in contrast to ES and maGSCs cells. It remains an open question whether somatic stem cells derived from the postnatal animal are pluripotent and whether truly pluripotent cells can be isolated from somatic tissues by expansion in culture (as can been done with unipotential PGCs or spermatogonial stem cells). At issue is whether somatic stem cells of tissues such as the hematopoietic system, the intestine or the skin that are multipotent and can generate all cell types in their respective

lineages in vivo are inherently plastic and capable of "transdifferentiation" into cell types of other lineages. Claims for cellular "plasticity" rest on two criteria: (1) in vitro differentiation to different cell types and (2) transplantation of the cells into blastocysts or postnatal mice to assess their ability to contribute in vivo to different tissues (Jaenisch and Young, 2008).
While it is possible that prolonged in vitro culture induces transdifferentiation and pluripotency, this has not been clearly proven.
In summary, pluripotency and spontaneous transdifferentiation of somatic cells remains an unproven concept. While unexpected transformation events may occur in somatic lineages, such events are exceedingly rare, are not a major force in physiological repair, and may simply be due to events such as cell fusion.

## 2.4. Reprogramming by defined factors

Lineage-associated transcription factors help to establish and maintain cellular identity during development by driving the expression of cell type-specific genes while suppressing lineage-inappropriate genes.
The fourth principle that contributed to the discovery of induced pluripotency was the observation that lineage-associated transcription factors can change cell fate when ectopically expressed in certain heterologous cells. Takahashi and Yamanaka recently achieved a significant breakthrough in reprogramming somatic cells back to an ES-like state (Takahashi and Yamanaka, 2006). They successfully reprogrammed mouse embryonic fibroblasts (MEFs) and adult fibroblasts to pluripotent ES-like cells after viral-mediated transduction of the four transcription factors Oct4, Sox2, c-*myc*, and Klf4. These cells were detected by using a reporter under the control of the *Fbx15 gene*, encoding for a protein expressed in undifferentiated embryonic stem cells, and named induced pluripotent stem

cells ("iPS"). IpS cells were shown to be pluripotent by their ability to form teratomas, although the first isolates were unable to generate live chimeras. Their pluripotency was dependent on the continuous viral expression of the transduced *Oct4* and *Sox2* genes, with the endogenous *Oct4* and *Nanog* genes being either not expressed or expressed at a lower level than in ES cells, and their respective promoters were found to be largely methylated. This is consistent with the conclusion that the Fbx15-iPS cells did not correspond to bona fide ES cells but may have represented an incomplete state of reprogramming. While genetic experiments established that Oct4 and Sox2 are essential for pluripotency (Chambers and Smith, 2004); (Ivanova et al., 2006); (Masui et al., 2007), the role of the two oncogenes c-*myc* and *Klf4* in reprogramming is less clear. When activation of the endogenous *Oct4* or *Nanog* genes was used as a more stringent selection criterion for pluripotency, the resulting Oct4-iPS or Nanog-iPS cells, in contrast to Fbx15-iPS cells, were fully reprogrammed to a pluripotent, ES cell state by molecular and biological criteria, including chimera formation and germline transmission (Maherali et al., 2007) (Okita et al., 2007) (Wernig et al., 2007).
Expression of the reprogramming factors in fibroblasts appears to initiate a sequence of stochastic events that eventually leads to a small fraction of iPS cells. This is supported by clonal analyses demonstrating that the activation of pluripotency markers can occur at different times after infection in individual mitotic daughter cells of the same infected fibroblast (Meissner et al., 2007). Thus, ectopic expression of Oct4, Sox2, c-*myc*, and Klf4 may trigger a sequence of epigenetic events such as chromatin modifications or changes in DNA methylation that eventually result in the pluripotent state of some infected cells but not others even though they carry the identical combination of proviruses. These experiments also suggested that the frequency of reprogramming increases with time, resulting in up to 0.5% of the input mouse embryonic fibroblasts ("MEFs") giving rise to iPS cells at 3 to 4 weeks after infection (Meissner et al., 2007). The original isolation of iPS

cells was based upon retrovirus-mediated transduction of four genes (two of which are oncogenes) and on drug-dependent selection for Fbx15, Oct4, or Nanog activation. These experimental conditions seriously hinder the eventual application of the in vitro reprogramming approach for therapeutic use in humans because mice derived from iPS cells frequently develop cancer (Okita et al., 2007) and because the isolation of human iPS cells cannot be based on genetically modified donor cells. Some of these limitations have been overcome in recent experiments. First, in an effort to reduce the risk of tumors in iPS cell-derived chimeras, more recent experiments showed that c-*myc* is dispensable for reprogramming (Nakagawa et al., 2008) (Wernig et al., 2008) (Yu et al., 2007), though the reprogramming process was significantly delayed and less efficient in the absence of this oncogene. While mice derived from these iPS cells will not develop c-*myc*-induced tumors (Nakagawa et al., 2008) (Wernig et al., 2008), it is not clear whether other retrovirus-transduced transcription factors, such as Oct4 (Hochedlinger et al., 2005), will cause tumors at later stages. Second, fully reprogrammed, genetically unmodified mouse fibroblasts were isolated based only on morphological criteria, without stringent selection for activation of a neomycin-resistance gene (Blelloch et al., 2007) (Meissner et al., 2007). Subsequent to these studies, human iPS cells were isolated from genetically unmodified fibroblasts (Takahashi et al., 2007) (Yu et al., 2007) (Park et al., 2008), indicating that combinations of factors similar to those used for reprogramming of mouse cells was also effective for human cells.

One of the promises of patient-specific ES cells is the potential for customized therapy of degenerative diseases. Previous studies have shown that disease-specific ES cells produced by nuclear cloning in combination with gene correction can be used to cure an immunological disorder in a proof-of-principle experiment in mice (Rideout et al., 2002). In a similar approach, it has been recently demonstrated that iPS cells derived from skin cells of a mouse with sickle cell anemia were able to restore normal blood

function when hematopoietic derivatives transplanted into diseased mice (Hanna et al., 2007).

## 3. Lineage Reprogramming

The fate of a cell can be altered by forced expression of single tissue-specific transcription factor. Gehring and colleagues were the first to show this in 1987 (Schneuwly et al., 1987): in *D. melanogaster* larvae, ectopic overexpression of a homeotic gene, *Antennapedia*, under the control of a heat-shock gene promoter led to a change in body plan, with an additional set of legs being formed instead of antennae. Even more striking was the finding by Gehring (Gehring, 1996) almost a decade later that ectopic expression of *eyeless* (known as *Pax6* in mice), a master controller of a cascade of 2,500 genes, led to the development of functional eyes on the legs, wings and antennae of *D. melanogaster*. In mice, the first tissue-specific master regulatory transcription factor was identified by Weintraub and colleagues (Davis et al., 1987) in 1987. They found that it was possible to induce a phenotypic conversion to the myogenic lineage by expressing a single muscle helix–loop–helix protein MYOD (Davis et al., 1987). Subsequently, Graf and colleagues (Xie et al., 2004), (Laiosa et al., 2006) discovered that primary B and T cells could be converted efficiently into functional macrophages upon overexpression of the myeloid transcription factor C/EBPα, and when the gene encoding the transcription factor PAX5 was removed from B cells, these cells reverted to less specialized progenitors (Cobaleda et al., 2007). More recently, researchers have identified sets of transcription factors that induce the conversion of pancreatic acinar cells into insulin-producing β cells by overexpressing the pancreatic factors MafA, Pdx1, and Ngn3 (Zhou et al., 2008); the conversion of muscle precursors into brown fat cells, by the overexpression of C/EBPβ and PRDM16 (Kajimura et al., 2009); the

reprogramming of fibroblasts into cardiomyocytes by the cardiac factors Gata4, Mef2c, and Tbx5 (Ieda et al.); the transdifferentiation of T cells into natural killer T (NK-T) cells, by the ablation of Bcl11b (Li et al.); and the conversion of fibroblasts into neurons by the activation of the neural factors Ascl1, Brn2, and Myt1l (Vierbuchen et al.) (Fig. 3).

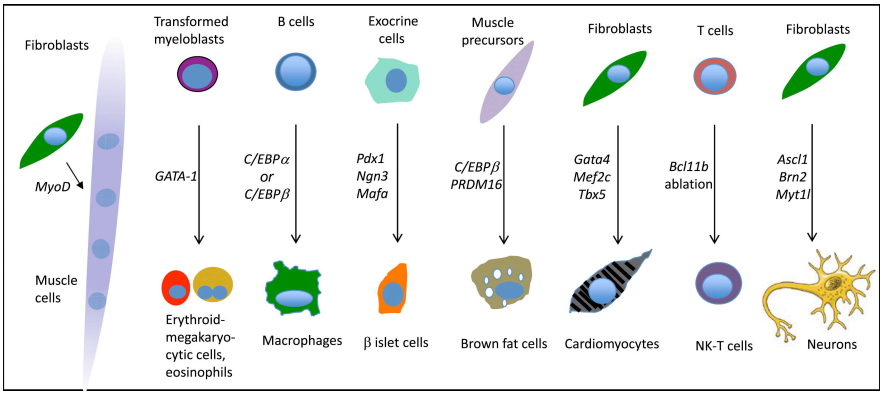


Figure 3. Examples of reprogramming by defined transcription factors. The examples shown are discussed throughout the text. Models (left to right) based on work from (Davis et al., 1987), (Kulessa et al., 1995), (Xie et al., 2004), (Zhou et al., 2008), (Kajimura et al., 2009), (Ieda et al., 2010), (Li et al., 2010), (Li et al., 2010) and (Vierbuchen et al., 2010). Adapted from (Graf, 2011)

Of note, these experiments proved that lineage conversions are not restricted to cell types within the same lineage or germ layer, since fibroblasts are mesodermal in origin, whereas neurons are derived from ectoderm. This suggests the possibility that any specific cell type may be directly converted into any other if the appropriate reprogramming factors are known. Direct lineage conversions could provide important new sources of human cells for modeling disease processes or for cellular-replacement therapies. For future applications, it will be critical to carefully determine the fidelity of reprogramming and to develop methods for robustly and efficiently generating human cell types of interest.

3.1. Reprogramming of B cells into macrophages

The conversion of cells from one hematopoietic lineage into another can be achieved by forced expression of lineage specific and instructive transcription factors. This is summarized within the lineage tree shown in Fig. 4.

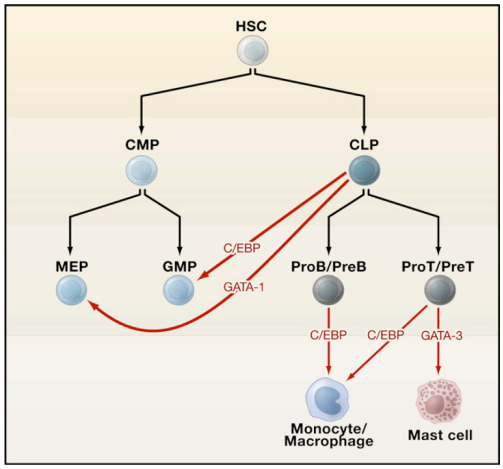


Figure 4. Reprogramming of hematopoietic lineages. The red arrows depict lineage reprogramming upon expression of the transcription factors GATA-1, C/EBP, or GATA-3. HSC, hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid Progenitor; GMP, granulocyte/macrophage progenitor. Adapted from (Orkin and Zon, 2008)

Previously, our group reported the reprogramming of primary B cells into macrophages by overexpression of C/EBPα in a fast (5 days) and highly efficient way (approximately 65% of pre-B cells and 35% of mature B cells) (Xie et al., 2004). In vitro reprogrammed cells resembled macrophages based on morphology and phagocytic capacity and moreover gene profiling reveled loss of lymphoid gene expression and upregulation of macrophage-specific genes. C/EBPα induces these changes by inhibiting the B cell commitment transcription factor Pax5, leading to the downregulation of its target CD19. It also synergizes with endogenous PU.1, an ETS family factor that is moderately expressed in B cells and highly expressed in macrophages, leading to the upregulation of its target Mac1 and other myeloid markers.

These two processes can be uncoupled since, in PU.1-deficient pre-B cells, C/EBPα induces CD19 regulation but not Mac1 activation.

Considering that the primary cell system is heterogenous, with a subset resistant to reprogramming, that the cultures require stroma cells and that cell numbers are limiting, we developed a robust transdifferentiation system, consisting of a clonal pre-B cell line expressing C/EBPα fused to the hormone-binding domain of the estrogen receptor (ER) (Bussmann et al., 2009). The C/EBPαER expressing B cells can be converted by β-estradiol, in the absence of stroma cells, into macrophage-like cells at essentialy 100% efficiency within 2 to 3 days. Using the inducible cell line it became possible to directly visualize the reprogramming process, to analize the methylation of specific promoters (Rodriguez-Ubreva et al.), and to test for genes with the capacity to accelerate or inhibit cell reprogramming.

## 4. The CCAATT/enhancer-binding protein α

C/EBPα is the prototypical basic-region leucine zipper (bZIP) transcription factor (Darlington et al., 1998). It belongs to a family of six members that is characterized by a leucine zipper that allows dimer formation and the presence of a basic region that mediates DNA binding. In addition to forming homodimers, C/EBPα dimerizes with other members of the C/EBP family (C/EBPβ, -γ, -δ, ε, and CHOP) (Lekstrom-Himes and Xanthopoulos, 1998).

A key role of C/EBPα is to regulate differentiation of a select set of cell types. Within the hematopoietic system, C/EBPα is expressed in myeloblast progenitors and granulocytes. Ectopic expression of C/EBPα in bipotential myeloid cells induces granulopoiesis and blocks monocyte differentiation (Radomska et al., 1998), whereas loss of C/EBPα results in an absence of granulocytes and macrophages (Zhang et al., 1997). C/EBPα is likewise

important for the formation adipose tissue, where it mediates differentiation of preadipocytes into adipocytes and regulates the metabolism of mature adipocytes (Darlington et al., 1998); (Rosen and Spiegelman, 2000). The requirement of C/EBPα for viability, however, is most dramatically revealed by its functions in the liver and lung. Without intervention, C/EBPα$^{-/-}$ mice die within hours of birth of hypoglycemia due to impaired function of hepatocytes (Wang et al., 1995) or of respiratory failure due to impaired function of type II pneumocytes (Flodby et al., 1996) (Linhart et al., 2001). C/EBPα is also expressed, although its function remains poorly characterized, in the intestine, adrenal gland, skin, mammary gland, placenta and brain (Lekstrom-Himes and Xanthopoulos, 1998).

Several recent studies highlight the crucial antimitotic role of C/EBPα, which inhibits cell growth through a variety of mechanisms. First, C/EBPα induces expression and stability of the cyclin-dependent kinase inhibitor, p21 (Timchenko et al., 1996). Second, C/EBPα interacts directly with the cyclin-dependent kinases Cdk2 and Cdk4 and blocks their ability to interact with cyclins, thereby impeding cell cycle progression (Harris et al., 2001) (Wang et al., 2002) (Wang et al., 2001). Finally, C/EBPα directly represses the activity of E2F, a key transcriptional regulator of cell cycle genes (Johansen et al., 2001) (Slomiany et al., 2000) (Timchenko et al., 1999a) (Timchenko et al., 1999b). Indeed, the ability of C/EBPα to repress E2F may be necessary for induction of differentiation to granulocytes and adipocytes (Johansen et al., 2001) (Porse et al., 2001). It has been reported though, that dephosphorylation of C/EBPα at Ser193 accelerates cell proliferation in the liver, suggesting a pathway by which the antimitotic function of C/EBPα can be switched off (Wang and Timchenko, 2005). C/EBPα therefore appears to be a good candidate for the study of the connection between cell cycle and cell differentiation.

## 5. Cell cycle, cell differentiation and reprogramming

Studies in a variety of systems indicate that cell division has a role in many aspects of organogenesis, not only in mediating growth and shape of organs, but also in the generation of cell diversity through asymmetric division, the timing of biological clocks, and the regulation of distances between signaling centers (Horvitz and Herskowitz, 1992) (Pourquie, 1998) (Tabin, 1998). Cell cycle progression and differentiation are usually seen as two distinct and mutually exclusive processes during development. When cells continue to cycle they do not differentiate, and when cells terminally differentiate they no longer cycle. It has been theorized that, for some cell types, the process of DNA replication remodels chromatin structure and allows access to previously inactive regulatory domains, thereby changing the expression patterns of specific genes in dividing cells, and making a "quantal mitosis" a requirement for differentiation decisions (Holtzer et al., 1975) (Holtzer et al., 1983).

In support of this theory, inhibition of cell proliferation in the chicken limb by various mechanisms cause alterations in gene expression and leads to diverse effects on limb morphology, such as supernumerary digits or the loss of proximal elements (Stephens, 1988) (Ohsugi et al., 1997). On the other hand, it has been shown that chicken myoblasts infected with a temperature-sensitive mutant of Rous sarcoma virus could differentiate into multinucleated myotubes in absence of cell proliferation (Falcone et al., 1984).

Experimental manipulation of cell cycle regulators permits to directly address causality when examining connections between the cell cycle and differentiation. Although a temporal coupling of cell cycle arrest and terminal differentiation is common during development, and therefore may seem obligatory, differentiated myocytes overexpressing the G1–S activator E2F1 incorporate bromodeoxyuridine (BrdU) into nuclei, indicating that entry into

the S phase can still occur in these cells (Chen and Lee, 1999). So differentiation does not necessarily preclude progress through the cell cycle. A number of recent experiments indicate that cell cycle arrest may be necessary for differentiation but is not always sufficient. For example, oligodendrocyte differentiation involves both changes in gene expression and concurrent cell cycle arrest. In order to determine whether cell cycle arrest is sufficient to cause differentiation, Tang et al. (Tang et al., 1999) inhibited proliferation of oligodendrocyte precursors in vitro by overexpressing p27, a well-known Cdk inhibitor. They discovered that the precursors stopped dividing, but did not express proteins associated with differentiation. This suggests that cell cycle arrest per se is not sufficient for differentiation, and that other signaling pathways are also needed.

Regarding the in vitro reprogramming of differentiated cells into iPS cells it is known that multiple DNA replication cycles and cell divisions are required. Previous studies have proposed several models for reprogramming (Yamanaka, 2009) (Hanna et al., 2009) including a "stochastic one-step model" (Hanna et al., 2009) whereby reprogramming of a given cell occurs stochastically in one step throughout the time line of the experiment at a uniform intrinsic probability per cell that depends only on the derivation conditions. Moreover, increased cell proliferation accelerates the kinetics of iPS cells formation (Kawamura et al., 2009) (Hong et al., 2009) (Banito et al., 2009) (Marion et al., 2009) (Hanna et al., 2009). Accelerated cell division could amplify the number of target cells and therefore the probability of becoming an iPS cell. Alternatively, DNA replication may be a prerequisite for epigenetic changes to occur, such as DNA and histone modifications, which allow the transitions to pluripotency.

The requirement for DNA replication in nuclear transfer reprogramming is not very clear. Although one ES cell–somatic cell fusion experiment suggested that replication is essential for reprogramming (Do and Scholer, 2004), nuclear transfer experiments indicated the presence of a replication-

independent mechanism, possibly involving an active DNA demethylase (Simonsson and Gurdon, 2004). The different results might be due to biological differences in the cell types (ES cell versus oocyte) and/or technical differences in the assays used (cell fusion versus nuclear transfer).

Finally, it has been shown that in some transdifferentiation systems cell division is not required (Zhou et al., 2008) (Vierbuchen et al., 2010). Thus, during the reprogramming of adult pancreatic exocrine cells to beta islet-cells only 3.2% of the cells incorporated BrdU, compared to 12.9% for endogenous beta-cells (Zhou et al., 2008). In the conversion of fibroblasts to neurons 14% of induced-neural cells incorporated BrdU and 2% when BrdU was added one day after induction of the reprogramming factors (Vierbuchen et al., 2010). This showed that most of induced-neural cells became post mitotic within 24 hours after transgene activation. So far, therefore, the role of cell cycle during differentiation and reprogramming is still controversial and differs according to the type of reprogramming.

# Aims

This project has been developed in the context of studying the mechanisms of reprogramming pre-B cells into macrophages. The main objectives of the project are:

- Understanding whether reprogramming requires retrodifferentiation to a precursor cell state or is a direct process

  The transdifferentiation of pre-B cells into macrophages induced by C/EBPα constitutes an ideal system to examine this question, as cells can be converted at essentially 100% efficiency within 3 to 5 days. In addition, the system offers the advantage that hematopoietic stem cells and various intermediate progenitor cells (HSPCs) are defined by specific cell surface antigen combinations and that expression array databases for these cells are available.

- Investigate whether DNA synthesis and cell cycle progression are necessary for cell reprogramming.

  We have previously developed a clonal pre-B cell line with an inducible form of the transcription factor C/EBPα that can be converted into macrophage-like cells. These cells double every 11 hours and once induced, switch at 100% efficiency in 2 to 3 days. Also because they require no feeder cells this system is ideal to study the role of cell cycle during reprogramming.

# PART II

# RESULTS

# Chapter 1

## C/EBPα induced transdifferentiation of pre-B cells into macrophages involves no overt retrodifferentiation

Alessandro Di Tullio, Thien Phong Vu Manh, Alexis Schubert, Robert Månsson, and Thomas Graf

*PNAS. October 11, 2011; 108 (41): 17016-17021*

# CCAAT/enhancer binding protein α (C/EBPα) induced transdifferentiation of pre-B cells into macrophages involves no overt retrodifferentiation

**Running Title:** No retrodifferentiation

Alessandro Di Tullio[1,3], Thien Phong Vu Manh[1,2,3], Alexis Schubert[1], Robert Månsson[4] and Thomas Graf[1, 5*]

[1] Cancer and Differentiation Program, Center for Genomic Regulation and UPF, Barcelona, Spain

[2] Present address: CIML, Luminy, France

[3] Shared co-authors

[4] Lund Strategic Research Center for Stem Cell Biology and Cell Therapy, Lund University, Lund, Sweden

[5] ICREA Research Professor

*Corresponding author

Center for Genomic Regulation, CRG

Carrer Dr. Aiguader 88

08003 Barcelona, Spain

Tel x 34 93 316 0127

thomas.graf@crg.es

# Chapter 2

## C/EBPα bypasses cell cycle-dependency during immune cell transdifferentiation

Alessandro Di Tullio and Thomas Graf

*Submitted. 2012 Apr 30.*

# C/EBPα bypasses cell cycle-dependency during immune cell transdifferentiation

**SHORT TITLE:** Cell cycle-dependency

Alessandro Di Tullio[1] and Thomas Graf[1, 2*]

[1]Gene Regulation, Stem Cells and Cancer Program, Center for Genomic Regulation (CRG) and Pompeu Fabra University. 08003 Barcelona, Spain and [2]Institucio Catalana de Recerca i Estudis Avanc ats (ICREA), Pg. Lluis Companys 23, 08010 Barcelona, Spain

*Corresponding author
Center for Genomic Regulation, CRG
Carrer Dr. Aiguader 88
08003 Barcelona
Spain
Tel x 34 93 316 0127
thomas.graf@crg.es

## ABSTRACT

Our earlier work has shown that pre-B cells can be converted into macrophage-like cells by overexpression of the transcription factor C/EBPα or C/EBPβ with high efficiency. Using inducible pre-B cell lines we have now investigated the role of cell division during C/EBP-induced reprogramming. The majority of cells reprogrammed by C/EBPα incorporated BrdU before arresting at G0 and all C/EBPβ induced cells incorporated the compound. This contrasts with reports from other systems where transdifferentiating cells essentially do not divide. Although inhibition of DNA synthesis led to an impairment of C/EBPα induced transdifferentiation, sorted G0/G1 and G2/M fractions showed no significant differences in their reprogramming kinetics. In addition, knocking-down p53 did not accelerate the transdifferentiation frequency, as it has been described for reprogramming of induced pluripotent (iPS) cells. Time-lapse experiments showed that after C/EBPα induction approximately 90% of cells divide once or twice, while 8% do not divide at all before acquiring a macrophage phenotype, supporting our BrdU incorporation results. Importantly, the non-dividing cell subset expressed the highest levels of C/EBPα and was the fastest in differentiating, suggesting that high levels of C/EBPα accelerate both the switching process and the cells' growth arrest. Our data show that traversing the cell cycle is not strictly required for pre-B cell to macrophage conversion and provide new evidence for the notion that the mechanisms of transcription factor induced transdifferentiation and iPS cell reprogramming differ.


## INTRODUCTION

Transcription factor–induced cell reprogramming has revolutionized the stem cell field. There has been an explosion in the last few years of reports describing direct transdifferentiation from one cell type to another. After the finding that MyoD can convert fibroblasts into muscle cells [1], numerous

other incidences of transdifferentiation have been reported, including that of immature and mature B cells into macrophages [2], committed T cell precursors into macrophages and dendritic cells [3], adult pancreatic exocrine cells into β-cells [4], and fibroblasts into neurons [5], cardiomyocytes [6], and hepatocyte-like cells [7] (see also review in [8]). In a separate line of work, Takahashi and Yamanaka have shown that it is possible to reprogram somatic cells into "embryonic stem cells" (so-called induced-pluripotent stem cells, iPSCs) by overexpressing a set of four transcription factors [9].

Mechanisms that link the cell cycle with differentiation or transdifferentiation are poorly understood and remain somewhat controversial. Cell-cycle progression and differentiation can be considered as two distinct and mutually exclusive processes during development: cells that are cycling typically do not differentiate, while cells that have terminally differentiated cease to divide. In the 70s and 80s, Holtzer et al. proposed that terminal differentiation requires a critical cell division ('quantal mitosis') [10]. Further, it was suggested that, during DNA replication, remodeling of chromatin allows access of regulatory factors to previously inactive regulatory domains [10],[11]. However, chicken myoblasts can be induced to differentiate into multinucleated myotubes in the absence of cell proliferation [12] and BrdU incorporation experiments have demonstrated that the majority of cells do not divide during transcription factor–induced transdifferentiation of pancreatic exocrine into endocrine cells, and of fibroblasts into neurons [4, 5]. On the other hand, increasing the cell division rate, such as by p53 or p21 knockdown, accelerates reprogramming of fibroblasts and of B cells into iPS cells [13],[14],[15].

Our earlier work has shown that overexpression of the transcription factors C/EBPα and C/EBPβ can induce the conversion of pre-B cells into macrophages [2]. The transdifferentiation induced by C/EBPα represents a direct transition from one cell type to another [16]. C/EBPα plays a dual role in myeloid differentiation and in cell cycle control. On the one hand, ablation

experiments in mice demonstrated that it is required for the formation and committment of myelomonocytic cells [17]. On the other hand, C/EBPα inhibits cell growth through a variety of mechanisms: it inhibits the interaction of the two cyclin-dependent kinases, Cdk2 and Cdk4, with cyclins [18], induces the expression of the Cdk inhibitor p21 [19], and represses the activity of the E2F transcription factor [20].

C/EBPα-induced transdifferentiation of pre-B cells into macrophages constitutes an ideal system to investigate the question of whether cell division is necessary for the transdifferentiation process [21]. Using this system, we found that the majority of the cells divide once or twice during reprogramming but that a small yet significant proportion does not divide. Those cells were found to be the first ones to transdifferentiate and to contain high levels of exogenous C/EBPα. We conclude that cell division is not required for the reprogramming of pre-B cells into macrophages and suggest that the cells that do divide represent a carry-over effect from the rapidly cycling pre-B cells.

## RESULTS

### C/EBPα induced pre-B cells undergo one cell division in average before arresting in G0 during transdifferentiation

To investigate the role of cell division during C/EBPα-induced reprogramming of pre-B cells into macrophages, we analyzed a pre-B cell line (C11) expressing C/EBPα-ER. This line can be converted at 100% efficiency into functional macrophage-like cells following treatment with β-estradiol (β-Est) [21]. We used carboxyfluorescein diacetate succinimidyl ester (CFSE) staining to monitor cell divisions. When CFSE diffuses into cells its acetate groups are cleaved by esterases, the compound becoming fluorescent and impermeable. Since CSFE is very stable, its fluorescence intensity decreases by approximately half with every cell division. As expected, C11 cells stained with CFSE proportionally decreased their

fluorescence intensity when maintained without induction (Fig. 1*A*, left), suggesting an approximate doubling time of 11 h. In contrast, when C11 cells were induced with b-Est, the CFSE fluorescence intensity decreased by half within the first 24 h and remained stable thereafter (Fig. 1*A*, right). Therefore, cells divided on average once before they arrested their proliferation after one day, confirming results obtained with another inducible cell line [21]. We next analyzed the cell cycle at different time points during transdifferentiation with propidium iodide (PI) staining. As shown in Figure 1B, the cells entered into the G0/G1 phase 24 h after induction, consistent with the known inhibitory effect of C/EBPα on cell proliferation. We further determined at which point C/EBPα blocks the cell cycle during reprogramming by using Ki67 staining. Our results show that at 24 h after induction, 40% of the cells were blocked in G0, and 57% in G1, but that at 48 h, all cells were in G0 (Fig. 1*C*). Finally, we analyzed gene expression arrays of transdifferentiating cells, obtained in our previous work (GEO number GSE17316)[21]. We found that many genes required for cell cycle progression, including *cyclinA* (*Ccna2*), *cyclinE* (*Ccne2*), *CDC2* (*Cdc2a*), and *cyclinB1* (*Ccnb1*), were dramatically downregulated during reprogramming (Fig. 1*D*). The significance of the observed transient upregulation of these markers is unclear. In conclusion, during C/EBPα induced transdifferentiation, pre-B cells divide on average once before arresting in the G0 stage.

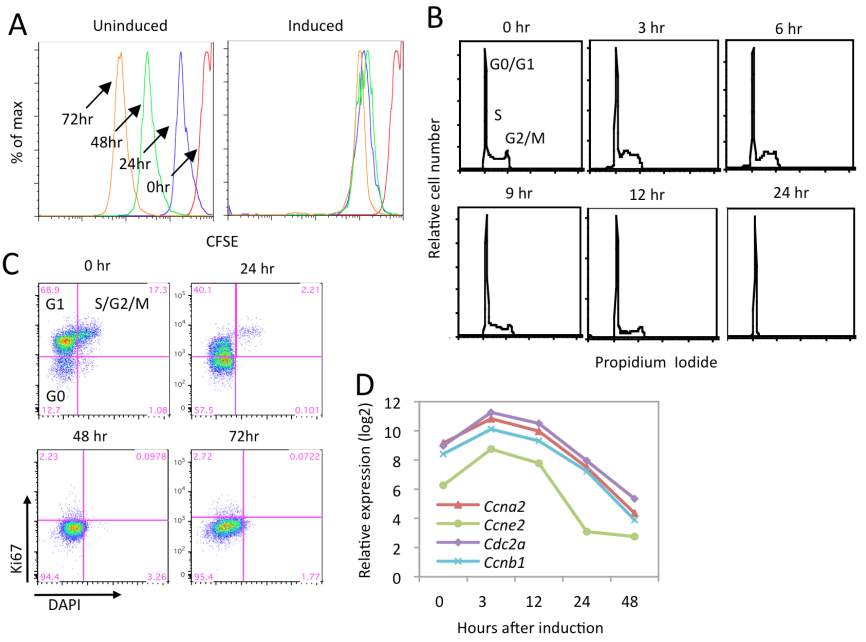


**Fig 1. Cell cycle analysis of pre-B cells during C/EBPα-induced transdifferentiation.** (A) Mean fluorescence intensity of CFSE of uninduced and C/EBPα-induced cells. The colors of the profiles on the right correspond to those indicated on the left. (B) Propidium iodide staining analysis of pre-B cells induced for different times. (C) FACS profiles of pre-B cells at different times of induction, monitoring Ki67 and DAPI expression. The fraction of cells corresponding to the different cell cycle stages are indicated. (D) Affymetrix array expression profiles of four cell cycle genes that were downregulated during reprogramming.

## A subset of pre-B cells induced by C/EBPα, but not C/EBPβ, transdifferentiated without exhibiting DNA synthesis

To determine whether all cells induced by C/EBPα pass through the S phase before converting into macrophages we studied 5-bromodeoxyuridine (BrdU) incorporation. When C11 cells were incubated with both b-Est and BrdU for 24 h and then analyzed by FACS, 90.5% of the cells were found to have incorporated the nucleoside analogue, while the rest were negative (Fig. 2*A*). In contrast, and as expected, uninduced C11 cells scored 100% positive for BrdU incorporation (Fig. 2*A*). Our earlier work showed that C/EBPβ, a transcription factor closely related to C/EBPα, is also capable of inducing

pre-B cell transdifferentiation, but without inducing withdrawal from the cell cycle [2]. To test the relationship between C/EBPβ-induced transdifferentiation and the cell cycle, we used a C11 cell line derivative (C11α/β) that contains, in addition to C/EBPαER, a doxycycline-inducible form of C/EBPβ. CSFE labeling experiments showed that the growth of C11α/β cells was arrested 2 days after treatment with 1 mg/ml doxycycline, at which time they partially upregulated Mac-1 and downregulated CD19 (Fig. S1).

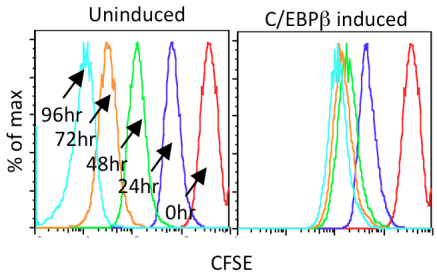


**Fig S1.** CFSE mean fluorescence intensity of uninduced cells (left) and C/EBPb induced cells (right).

BrdU incorporation showed that 100% of the C/EBPβ cells went through S phase (Fig. 2*B*).

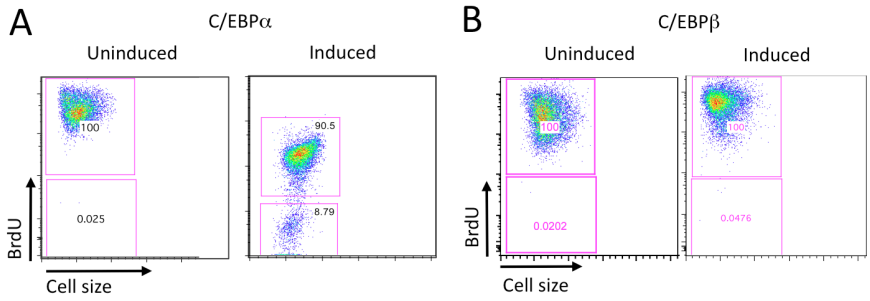


**Fig 2. DNA synthesis during C/EBPα- and C/EBPβ-induced pre-B cell to macrophage conversion.** (A) FACS profiles of BrdU incorporation in uninduced C11 cells and cells 24 h after induction with C/EBPα. (B) FACS profiles of BrdU incorporation in uninduced C11 cells and cells 24 h after induction with C/EBPβ.

Together, these results show that the vast majority of pre-B cells induced to differentiate by C/EBPα and C/EBPβ go through S phase, raising the possibility that transdifferentiation requires cell division.

**Inhibition of cell division blocks establishment of the myeloid program**

Next we tested the effect of cell cycle inhibitors on transdifferentiation. We used aphidicolin as a highly specific DNA synthesis inhibitor that blocks DNA polymerase α. Since we found that effective concentrations induced cell death we decided to create a C11 derivative that overexpresses the anti-apoptotic gene Bcl2 (C11-Bcl2). Treating these cells with 20μM aphidicolin for 48 h caused an almost complete block of cell proliferation, as judged by CFSE staining (Fig. 3A). We therefore pre-incubated the cells for 48 h with aphidicolin and then induced them with β-Est, while maintaining the treatment with the DNA synthesis inhibitor. Subsequent FACS analyses showed that Mac-1 upregulation was inhibited in the presence of aphidicolin, but that CD19 downregulation was not affected or even slightly accelerated (Fig. 3B). The inhibition of the macrophage program could also be detected by a lack of morphological changes, an inhibition of cell size and of granularity characteristic of macrophages. Furthermore, in the presence of aphidicolin, the phagocytic capacity was reduced from >90% in the control Mac1+/CD19- cells to about 30% (Fig. 3C).

Similar findings were made when C/EBPα-induced cells were treated with another DNA synthesis inhibitor, mitomycin C, as well as with the mitosis inhibitor monastrol. Together, these results suggest that the bulk of pre-B cells induced by C/EBPα need to traverse the cell cycle to effectively activate the macrophage program, whereas extinction of the B cell program, as determined by CD19 expression, is not diminished in the absence of DNA synthesis.

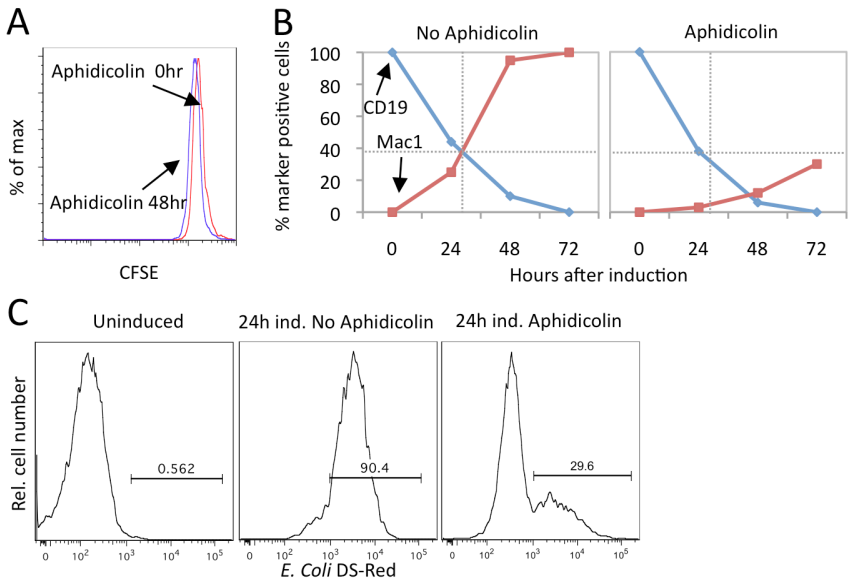


**Fig 3. Effect of aphidicolin on the transdifferentiation of pre-B cells.** (A) CFSE fluorescence intensity of uninduced C11-Bcl2 cells treated with the DNA synthesis inhibitor aphidicolin showing absence of cell division after 48 h. (B) Kinetics of CD19 downregulation and Mac1 upregulation during reprogramming of pre-B cells in the absence and presence of aphidicolin. (C) Effect of aphidicolin on the phagocytic capacity of induced C11-Bcl2 cells. Uninduced cells and cells induced for 72 h were grown in the presence or absence of aphidicolin and incubated with E. coli expressing dsRed prior to FACS analysis.

## The cell cycle stage of pre-B cells does not influence the reprogramming kinetics

The above findings suggested that DNA synthesis or another cell cycle checkpoint is required for transdifferentiation. To test this possibility further, we first asked whether transdifferentiation kinetics is influenced by the cell cycle stage of the starting population. The idea was that if transdifferentiation of pre-B cells into macrophages is dependent on cell cycle, then reprogramming kinetics of the two phases should be different, with G0/G1 cells reprogramming being substantially faster than G2/M cells, since these cells first have to go through M and G1 before reaching S phase (Fig. 4A). To sort C11 cells into G0/G1 and G2/M cell subsets, we used staining with Vybrant Ruby (Fig. 4B), a non-toxic DNA dye used for cell

cycle analyses in living cells. Reanalysis of sorted fractions confirmed that the two populations were well separated (Fig. 4C). Cells were then induced with β-Est, in parallel to unsorted control C11 cells. We found that the transdifferentiation kinetics, as determined by downregulation of CD19 and upregulation of Mac1, were indistinguishable between cells in G0/G1 phase (Fig. 4D, top) and those in G2/M phase (Fig. 4D, bottom).

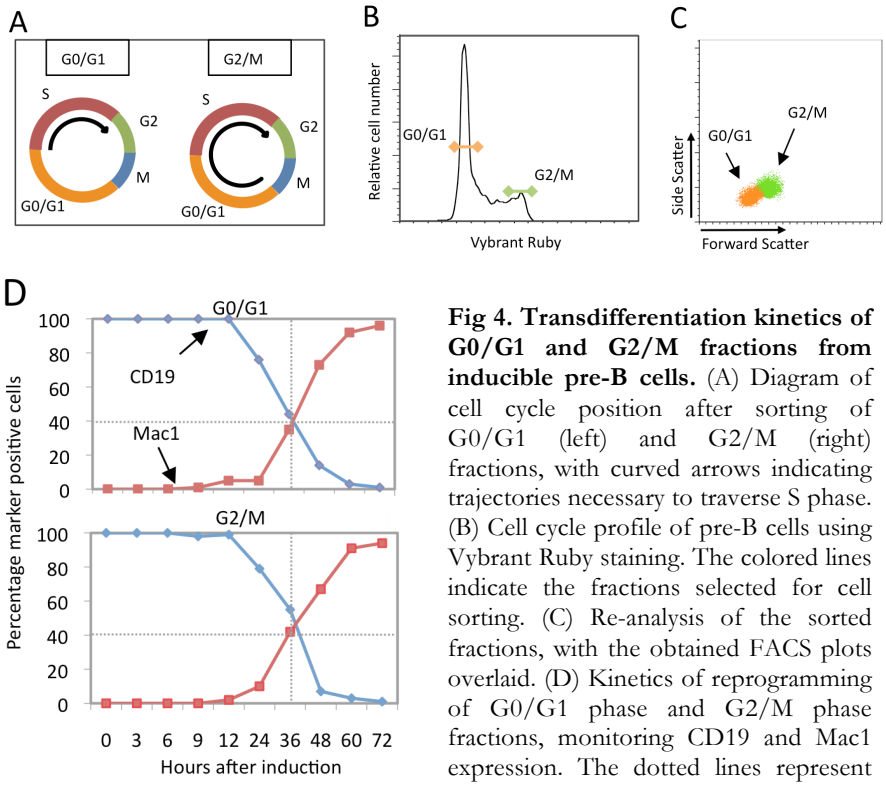


**Fig 4. Transdifferentiation kinetics of G0/G1 and G2/M fractions from inducible pre-B cells.** (A) Diagram of cell cycle position after sorting of G0/G1 (left) and G2/M (right) fractions, with curved arrows indicating trajectories necessary to traverse S phase. (B) Cell cycle profile of pre-B cells using Vybrant Ruby staining. The colored lines indicate the fractions selected for cell sorting. (C) Re-analysis of the sorted fractions, with the obtained FACS plots overlaid. (D) Kinetics of reprogramming of G0/G1 phase and G2/M phase fractions, monitoring CD19 and Mac1 expression. The dotted lines represent the crossing points of the CD19/Mac1 kinetics of the G0/G1 fraction.

Our earlier work showed that a pulse induction of 12 h on C11 pre-B cells, followed by washout of the inducer, was sufficient to convert a significant proportion of cells into macrophages [21]. We therefore repeated the above experiments using a 12-h β-Est pulse induction, instead of a continuous induction protocol, on the two sorted cell cycle stages. This assured that most of the induced cells have gone through one cell cycle, and that

exogenous C/EBPα did not cause a continuous inductive pressure. However, even under these conditions the kinetics of Mac1 upregulation was found to be indistinguishable between the G0/G1 and the G2/M fractions (Fig. S2A).

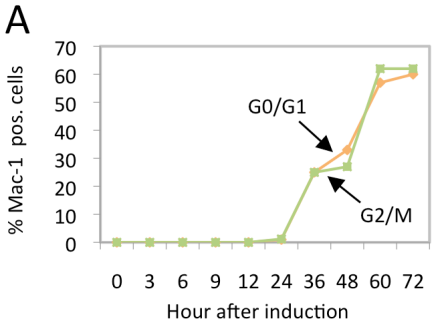


**Fig S2. Reprogramming of pre-B cells sorted in different phases of the cell cycle and in presence of the shp53.** (A) Kinetics of Mac1 upregulation of the G0/G1 subset (yellow line) and of the G2/M fraction (green line) after a 12 h pulse induction of C11 cells, followed by washout of the inducer.

Several groups have shown that p53 ablation accelerates the kinetics of induced pluripotent stem (iPS) cell formation, and that this acceleration is directly proportional to the increase in cell proliferation caused by inhibition of the tumor suppressor gene [13-15, 22]. We therefore tested whether a p53 short hairpin RNA (shp53) likewise accelerates the kinetics of B cell to macrophage transdifferentiation in our system. To this end, we infected C11 cells with a lentivirus containing shp53 and created a stable cell line (C11-shp53) in which the levels of p53 mRNA were decreased by 60% (Fig. S2B). C11-shp53 cells treated for 2 h with BrdU showed almost a threefold increase in the incorporation of the nucleoside analogue compared to C11 cells (Fig. S2C).

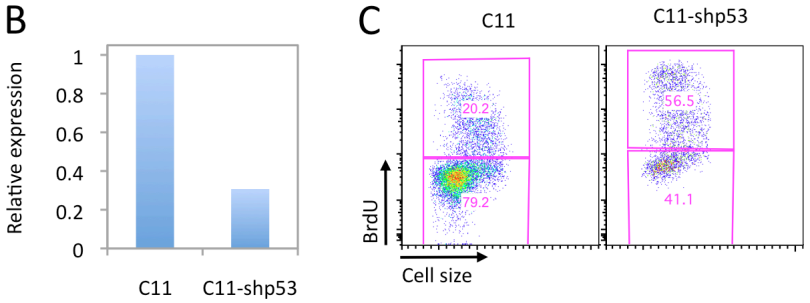

**Fig S2. Reprogramming of pre-B cells sorted in different phases of the cell cycle and in presence of the shp53.** (B) qRT-PCR expression values of p53 in C11 and C11-shp53 cell lines. (C) 2 h BrdU incorporation in C11 and C11-shp53.

Nevertheless, after inducing transdifferentiation, we found no acceleration of Mac1 upregulation or CD19 downregulation (Fig. 4E).

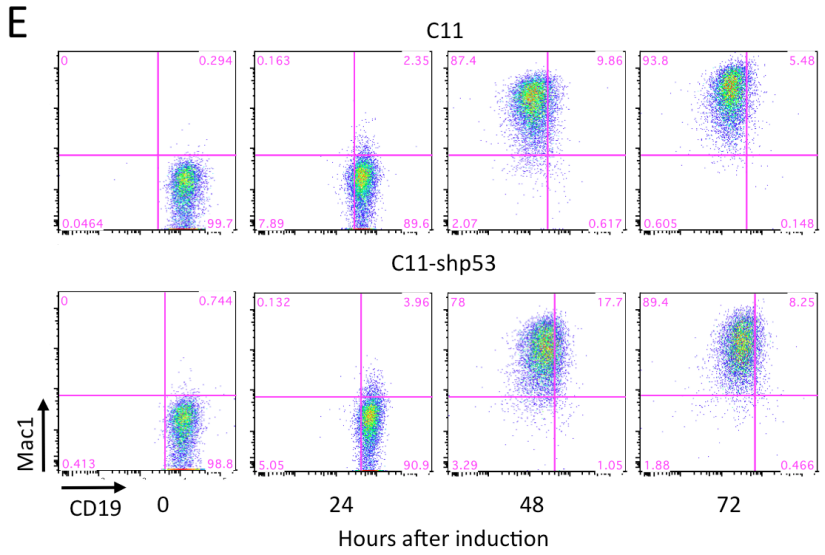


**Fig 4. Transdifferentiation kinetics of G0/G1 and G2/M fractions from inducible pre-B cells.** (E) FACS profiles of Mac1 and CD19 expression during reprogramming of pre-B cells in absence (top) or presence (bottom) of a short hairpin RNA against the p53 gene.

We also tested whether cell status changed during reprogramming of C11-shp53 cells, using Ki67 antibody staining. Two days after induction, all the cells were in G0 (Fig. S2D), exactly like control cells without shp53.

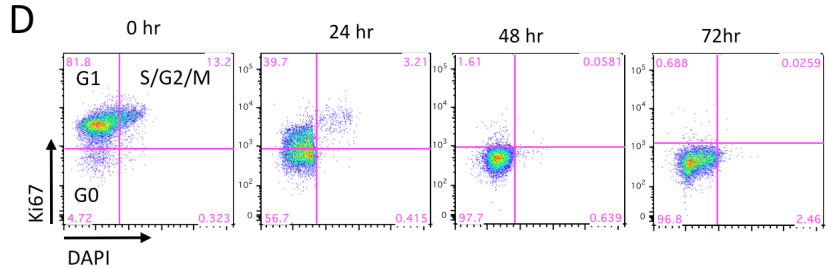

**Fig S2. Reprogramming of pre-B cells sorted in different phases of the cell cycle and in presence of the shp53.** (D) FACS plots of Ki67 and DAPI expression during pre-B cell to macrophage reprogramming in presence of a short harpin RNA against the p53 gene.

We therefore conclude that, unlike reprogramming of somatic cells into iPS cells, ablation of p53 does not accelerate the conversion of pre-B cells into macrophages nor does it prevent cell cycle arrest induced by C/EBPα. Taken together, these results indicate that neither G1/S nor G2/M transitions represent essential checkpoints for reprogramming.

## Time-lapse experiments reveal a subset of rapidly transdifferentiating cells that do not require cell division

To investigate the apparent discrepancy that DNA synthesis inhibitors can impair transdifferentiation yet traversing the S phase is not required for induced transdifferentiation, we first performed time-lapse experiments. Our earlier work with this technique showed that when pre-B cells transdifferentiate into macrophages, they become irregular in shape and are highly motile as early as 15 h after induction [21]. To address the question of whether all cells divide during C/EBPα-induced reprogramming, we therefore performed time-lapse experiments of C11 cells after induction with β-Est. We found that 5.8% of the induced pre-B cells divided twice, 86.4% divided once, and 7.8% of did not divide at all, before becoming motile and changing their morphology (Figs. 5A and S3A).

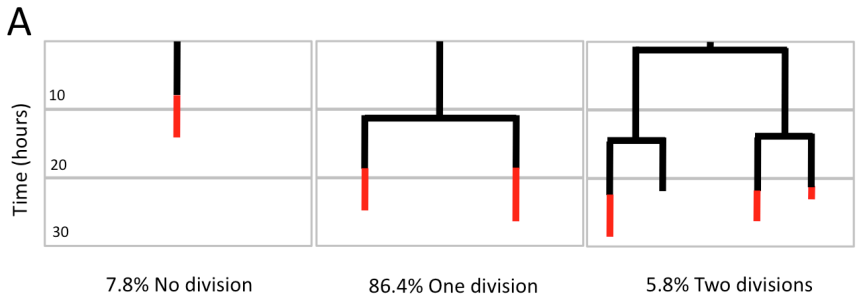


**Fig 5. Time-lapse experiments and effects of C/EBPα dosage on transdifferentiation of pre-B cells.** (A) Time-lapse microscopy analysis of

transdifferentiating cells, on a time scale from top to bottom. Results are shown from individual cells that did not divide (left), divided once (middle) or divided twice (right). The black bars indicate no differentiation, and the red bars, differentiation as determined by increased motility and acquisition of an irregular cell shape. The percentage of cells in each category is indicated.

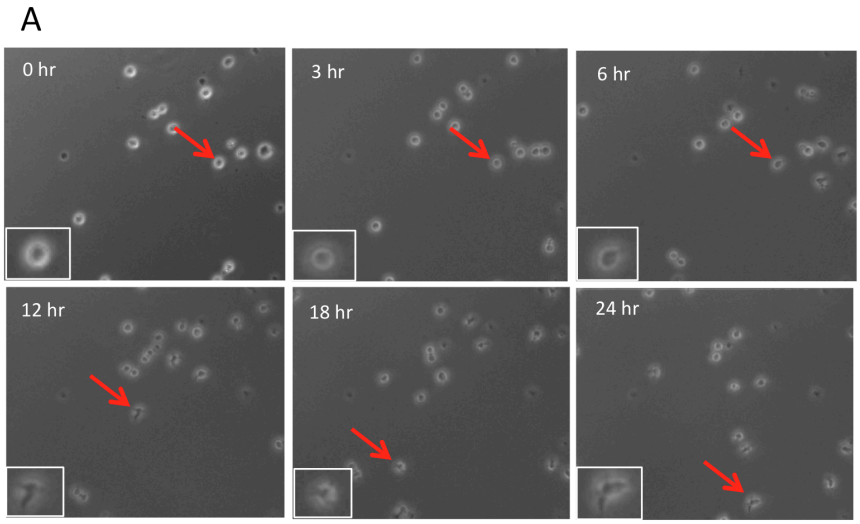


**Fig S3. Requirement of cell division during C/EBPα induced reprogramming of pre-B cells and C/EBPβ dosage dependency. (**A) Time-lapse microscopy analysis of pre-B cells during the first 24 hours of C/EBPα induced reprogramming. The red arrows show the different positions of a cell that transdifferentiated without division, as evidenced by a change in morphology (see enlarged image in insets) and increased motility from 12 hr post induction onwards.

Importantly, there were significant differences in the timing of transdifferentiation between these three groups of cells: cells that did not divide transdifferentiated into macrophage-like cells as early as after 8 h, while cells that divided once transdifferentiated first after 17 h, and cells that divided twice, after 23 h (Fig. 5B).

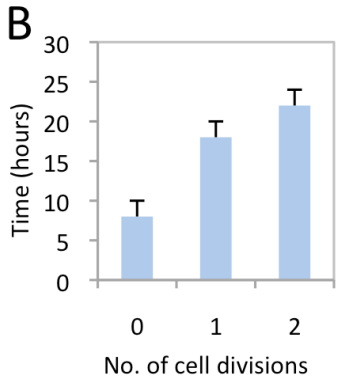


**Fig 5. Time-lapse experiments and effects of C/EBPα dosage on transdifferentiation of pre-B cells.** (B) Correlation of the time required for differentiating with the number of cell divisions.

It also became apparent that in most cases, the two daughter cells transdifferentiated at about the same time. These results confirm the finding that a small subset of induced C11 cells do not incorporate BrdU, supporting the notion that cell divisions are not strictly required for reprogramming.

**Pre-B cells that transdifferentiate without cell division express the highest C/EBPα levels**

Previous work has shown that high levels of C/EBPα induce the transdifferentiation of a higher percentage of primary pre-B cells than lower concentrations of the factor [2]. Therefore, the observed positive correlation between the speed of transdifferentiation and of the proportion of cells that did not divide raised the possibility that the most rapidly switching cells contained higher levels of C/EBPα. To test this, we performed a BrdU incorporation experiment with C11 cells induced with β-Est and determined the relative C/EBPα levels, as indicated by the fluorescence intensity of GFP, by gating BrdU-negative (R1) and -positive cells (R2) (Fig. 5C, left). BrdU negative cells were significantly enriched in the GFP high fraction (Fig. 5C, right).

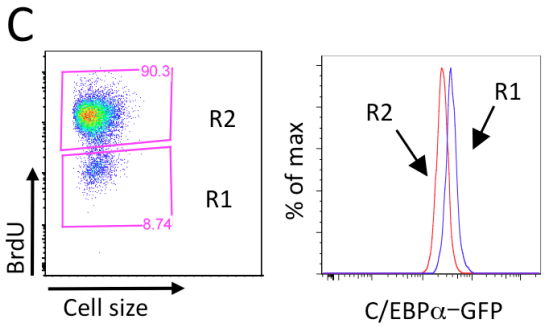


**Fig 5. Time-lapse experiments and effects of C/EBPα dosage on transdifferentiation of pre-B cells.** (C) FACS profiles monitoring BrdU incorporation in C11 cells 24 h after induction (R1 and R2, BrdU negative and positive cells, respectively, on the left), with C/EBPα-GFP expression in R1 versus R2.

In addition, the fraction of cells that did not incorporate BrdU increased from 3% to 28% in C/EBPα low and high cells, respectively (Fig. S3B).

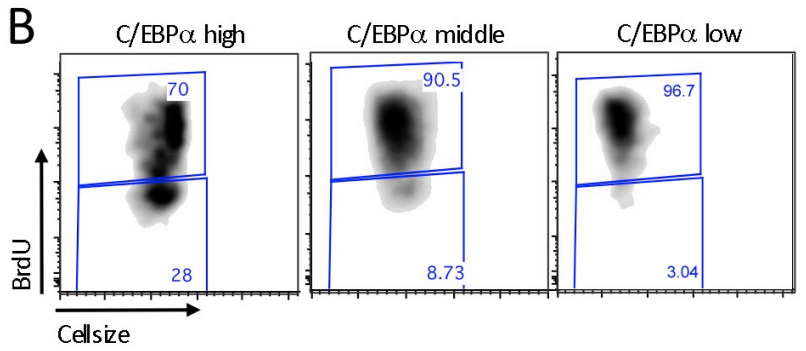


**Fig S3. Requirement of cell division during C/EBPα induced reprogramming of pre-B cells and C/EBPβ dosage dependency.** (B) BrdU incorporation in pre-B cells 24 h after induction of reprogramming with high, medium and low levels of C/EBPα.

These results indicate that high levels of C/EBPα inhibit cell division more effectively than lower levels. Next, we tested whether the rate of Mac-1 upregulation was affected by C/EBPα levels. Indeed, as shown in Figure 5D (left), cells with high amounts of C/EBPα upregulated Mac-1 expression much more rapidly than C/EBPα-medium or -low cells. In contrast, the rate of CD19 downregulation was essentially not affected (Fig. 5D, right).

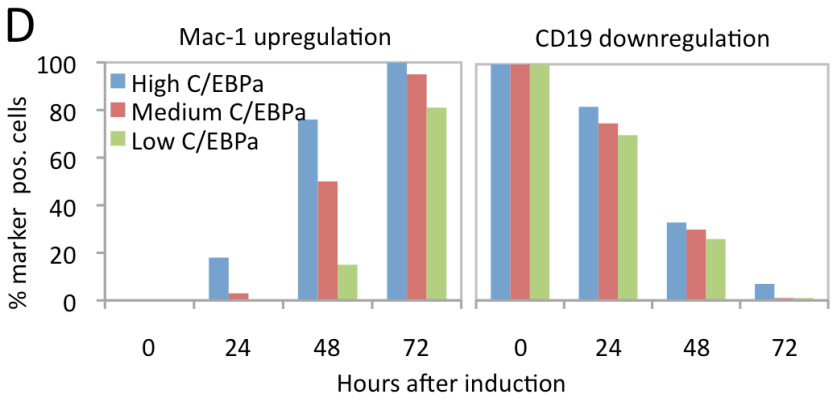


**Fig 5. Time-lapse experiments and effects of C/EBPα dosage on transdifferentiation of pre-B cells.** (D) Kinetics of reprogramming at different times of induction. Gated cells with high, medium, and low levels of C/EBPα, as monitored by Mac-1 upregulation (left) and CD19 downregulation, are shown (right).

Similar findings were also made with C/EBPβ-induced cells (Fig. S4).

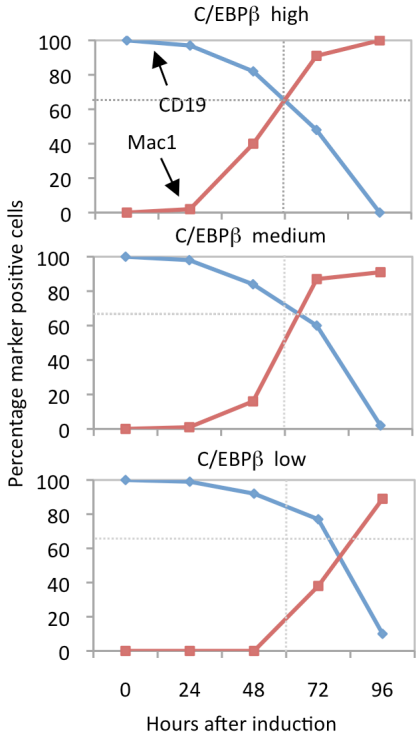


**Fig S4. C/EBPβ dosage dependency during C/EBPβ induced reprogramming of pre-B cells.** Kinetics of reprogramming at different times of induction with high (top), medium (middle) and low (bottom) levels of C/EBPβ, monitoring CD19 and Mac-1 expression.

These experiments therefore reveal that the transdifferention of pre-B cells to macrophages is accelerated by high levels of C/EBPα/β, and that in the case of C/EBPα, this leads to a decrease in the proportion of the cells that traverse the cycle.

## DISCUSSION

Our results have shown that during C/EBPα-induced transdifferentiation of pre-B cells into macrophages the majority of cells divide at least once, and that blocking DNA synthesis dramatically impairs transdifferentiation. However, the rate of transdifferentiation was independent of whether the starting cells were in G0/G1 or G2/M and a knockdown of p53, a treatment that accelerated the cells' growth rate, had no detectable effect on the transdifferentiation kinetics. In addition, approximately 10% of cells did not divide, as shown by BrdU incorporation and time-lapse experiments, indicating that cell division is not strictly required for reprogramming in this system. The finding that the non-dividing cells correspond to the most rapidly transdifferentiating cell subset, and that this subset expresses the highest levels of C/EBPα, suggests that they were forced to withdraw from the cell cycle by C/EBPα. These conclusions are indirectly supported by the findings with the transcription factor C/EBPβ: here pre-B cells converted into macrophages show a delay in cell cycle withdrawal compared to C/EBPα. Therefore, as expected, all transdifferentiating cells incorporate BrdU.

Our observation that immune cell transdifferentiation requires no cell division is in agreement with the finding that there are no significant promoter DNA methylation changes during transdifferentiation of pre-B cells into macrophages, with macrophages essentially maintaining the overall methylation pattern seen in pre-B cells [23]. This suggests that no major changes in DNA methylation are required for cell switching although it would be interesting to know whether inhibition of DNA methylation

modulates the switching rate. In apparent contrast to transdifferentiation DNA demethylation appears to play a critical role during iPS cell reprogramming [24-26], although it is controversial whether this requires DNA synthesis. Our findings also broadly confirm and extend conclusions reached in studies where fibroblasts were converted into neurons and pancreatic exocrine into endocrine cells [4, 5]. The major difference to our results is that in these systems the vast majority of cells do not incorporate BrdU during transdifferentiation. A plausible interpretation is that only a low proportion of primary fibroblasts and endocrine cells incorporate the compound, and so the induced conversions represent transitions between two essentially quiescent cell populations. In contrast, the switching system studied here represents a transition between rapidly proliferating cells (C11 pre-B cells divide approximately every 11 hours) and macrophages arrested in G0. It therefore appears that during C/EBPα-induced transdifferentiation, the lymphoid cells exhibit a 'carry over' effect, consisting of one or two rounds of cell division, before turning into quiescent macrophages.

The observation that the DNA synthesis inhibitor aphidicolin impaired C/EBPα-induced transdifferentiation was unexpected and is in apparent conflict with our finding that the S phase itself does not appear to represent a checkpoint required for myeloid gene activation. A possible explanation is that C/EBPα-induced cells prevented from cycling accumulate an unknown inhibitor of a myeloid regulator or fail to accumulate sufficient levels of a hypothetical co-factor.

The fact that the C/EBPα-induced cells were not prevented by shp53 from arresting in GO suggests that the cell cycle inhibitory effect of C/EBPα is dominant over the growth accelerating effect of shp53. Whether this plays out at the level of transcriptional regulation of the cell cycle inhibitor p21, a direct target of both C/EBPα and p53 [19],[27], remains to be determined. Our observation that a p53 knockdown does not alter the transdifferentiation kinetics of pre-B cells into macrophages contrasts with

its accelerating effects on iPS cell reprogramming (14-16). This difference lends further support to the postulate [28] that the mechanisms of transcription factor–induced transdifferentiation and iPS cell reprogramming are fundamentally different.

## METHODS

**Cells and viral constructs.** The construction of MSCV C/EBPαER IRES hCD4 virus and the creation of the C11 cell line was as described [26]. The Bcl2 cDNA was taken from pSFFV-Bcl2 (Addgene) and cloned into the pMSCV-puro with EcoRI digestion. The cell line C11-Bcl2 was generated by infecting C11 cells with the pMSCV-Bcl2-Puro retrovirus, selecting with puromycin, and sorting single cells. The shp53 lentivector was a kind gift of Dr Bill Keyes. The cell line C11-shp53 was generated by infecting C11 cells with the pMSCV-shp53-Puro-GFP lentivirus, selecting with puromycin, and sorting single cells. The pHAGE-tetO-C/EBPβ-IRES-tdTomato virus was constructed by inserting C/EBPβ cDNA in the pHAGE vector kindly provided by Dr. Gustavo Mostoslavsky [34], using NotI and BamHI. To generate the C11α/β line, C11 cells containing rtTA were infected with tetO-C/EBPβ tdTomato virus, and a single cell derived clone selected.

**Cell reprogramming, cell cycle inhibition, and FACS analyses.** C11 cell lines were induced with 100 nM β-Est (Calbiochem) and grown in special induction medium containing IL-3 and mCSF-1 (10 ng/mL) (Peprotech). The C11α/β cell line was induced with 1 μg/μL doxycyclin (Sigma). For the pulse induction experiment, cells were thoroughly washed and then incubated with 10 μM of the β-Est antagonist ICI (Tocris Bioscience). For the cell cycle inhibition experiments, cells were treated for 2 days with 20 μM aphidicolin (Calbiochem). Antibodies to cell surface antigens were purchased (BD PharMingen). Cells were analyzed with a FACS LSRII flow cytometer

(BD Biosciences) using FlowJo software (Tree Star, Ashland, OR) and sorted with a FACS ARIA flow cytometer (BD Biosciences).

**Real-Time PCR.** qRT-PCR reactions were carried out in triplicate as described [21]. Ct values were normalized to glucuronidase beta (GusB), and the relative expression was calculated by the Pfaffl method [30].

**Cell cycle analysis.** Cell cycle analyses were performed with the following different techniques. For CFSE staining, 3 × 105 cells were resuspended in 1 mL PBS with 0.2 μM CFSE for 5 min (Invitrogen) 4 h before the induction and then rinsed twice with 4 volumes of PBS. For propidium iodide staining, 2 × 105 cells were resuspended in 1 mL of cold 70% ethanol, stored overnight at 4°C, and then rinsed with PBS and stained for 15 min on ice with 300 μL cell cycle solution of 50 μg/mL PI (Sigma), 0.1 mg/mL RNaseA (Sigma) in PBS. For Ki67 staining, 2 × 105 cells were resuspended in 100 μl of Fix and Perm Medium A (Invitrogen), incubated for 15 min at room temperature, and rinsed with PBS. This was then resuspended in 100 μL of Fix and Perm Medium B (Invitrogen) with 5 μL of anti-Ki67-PE (BD Biosciences), incubated for 20 min at room temperature, rinsed with PBS, resuspended in 500 μL of PBS containing 5 μg/mL RNaseA and 2 μg/mL DAPI, incubated for 15 min at room temperature, and FACS analyzed. For BrdU staining, cells were treated for 24 h with 50 μM BrdU (2 × 105 cells in 1 mL of growth medium), resuspended in 100 μL of Fix and Perm Medium A (Invitrogen), incubated for 15 min at room temperature, rinsed with PBS, and resuspended in 100 μL of Fix and Perm Medium B (Invitrogen). Following another 20 min incubation at room temperature, cells were rinsed with PBS, resuspended in 100 μL of PBS with 10 μL of 10× DNaseI buffer (100 mM Tris-HCL, 25 mM MgCl2, 5 mM CaCl2) and 2 μL of DNase I solution (Promega 22U/μL), and incubated 1 h at 37°C in the dark. They were then resuspended in 100 μL PBS with 5 μL anti-BrdU-PerCP-Cy5.5

(BD Biosciences), rinsed, and FACS analyzed. For Vybrant Ruby staining, 2 × 106 cells were resuspended in 2 mL of growth medium with 4 mL of Vybrant Ruby (Invitrogen), incubated 1 h at 37°C in the dark, and centrifuged; cells were kept in 1 mL of the same solution in which they were stained.

**Time-lapse.** Microscopy chamber wells (μ-Slide 8 well coated poly-L-lysine from IBIDI) were pre-treated with 200 μL retronectin (Takara) (48 μg/mL) for 2 h at room temperature. Retronectin was removed, and 200 μL PBS, 2% BSA was added for 30 min. Wells were then rinsed with 300 μL PBS and left overnight in the incubator. 6 × 103 cells were resuspended in 200 μL of growth medium (with or without β-Est, IL3, and mCSF1) and added to the wells. Pictures were taken every 5 min (using 4 positions per well).

**Phagocytosis Assay.** C11-Bcl2 cells were seeded into 6-well plates, treated with 20 μM aphidicolin for 2 days, and then induced with β-Est for 3 days. 100 dsRed E. coli per cell were added, and plates were centrifuged at 800 × g for 15 min. Thereafter, cultures were incubated with 400 μg/mL gentamycin for 3 h at 37°C to eliminate extracellular bacteria. To remove excess bacteria from cells, a 2-mL suspension was underlayered with 5 mL of fetal calf serum and kept at room temperature for 2 h. Cells were collected from the serum phase, centrifuged, and analysed by FACS.

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# PART III
# DISCUSSION AND CONCLUSIONS

# Discussion

Our results have shown that C/EBPα induced transdifferentiation of pre-B cells into macrophages involves no overt retrodifferentiation and does not strictly require cell division. These data broadly confirm and extend conclusions reached in other systems (Zhou et al., 2008) (Vierbuchen et al.) (Ieda et al.), and therefore appear to be general principles of transdifferentiation which set it apart from iPS cell reprogramming.

1. No retrodifferentiation

At the outset of the work relative to the retrodifferentiation, we postulated different scenarios of transient progenitor gene reactivation during pre-B cell reprogramming into macrophages. We expected to either see no reactivation of progenitor genes, or transient reactivation of gene expression programs corresponding to specific cell stages (Fig. 5).

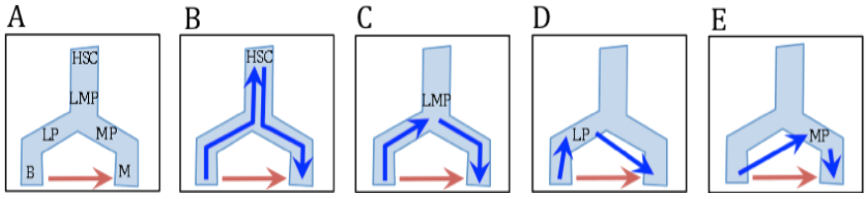


Figure 5. Scenarios of progenitor gene reactivation during reprogramming. The fork represents the bifurcation during hematopoiesis between B cells (B) and macrophages (M) that originate from hematopoietic stem cells, lymphoid myeloid-, lymphoid- or myeloid progenitors (HSC, LMP, LP, MP). The red arrow illustrates the induced reprogramming; the blue arrows the reactivation of stage specific gene expression programs. A, direct conversion without progenitor gene reactivation; B to E, transient reactivation of HSC genes (B); lymphoid/myeloid (LMP) progenitor genes (C); lymphoid progenitor (LP) genes (D); and myeloid precursor (MP) genes (E).

Two main findings indicate that C/EBPα induced reprogramming involves no overt retro-differentiation. First, none of the cell surface antigens commonly used to define HSCs and multipotent progenitors, including CD150, c-Kit, CD34, Flt3 and IL-7R, were reactivated. Second, a time-

restricted activation of C/EBPα did not induce a burst of hematopoietic colony formation. Thus, activating C/EBPα for 6, 12, and 24 hours by β-estradiol treatment followed by washout of the inducer and seeding of the cells in methylcellulose cultures containing SCF, IL-3, IL-6, EPO and TPO, conditions permissive for multipotent and more restricted hematopoietic progenitors, only yielded clusters of 2 to 4 cells, and these exhibited a macrophage morphology (data not shown).

Strikingly, genes restricted to multipotent progenitors (*Kit, Flt3 and Il7r*) were reactivated only at the transcriptional level. Why early progenitor RNAs are not translated into proteins is not clear. One possibility is that the mRNAs are down regulated before the translation machinery becomes active. Another is that cells undergoing reprogramming express translational inhibitors, such as micro RNAs, not present in normal hematopoietic precursors. No matter what explanation is correct our observations raise the possibility that the translation/protein export machinery differs between early hematopoietic progenitors and more restricted cell stages.

It is possible that only a subset of pre-B cells reactivates early progenitor genes. The finding that the induced expression of *Kit* and *Flt3* does not reach the levels observed in normal progenitors would support this idea, as does the observation that individual cells take different paths in the timing of macrophage program activation and B cell program extinction. However, it is still possible that all cells show low-level gene reactivation of multipotent progenitor markers. In contrast, the high expression of the more restricted myeloid progenitor markers reached late during reprogramming suggests that these genes are uniformly activated in all cells before the cells acquire their final fate. Our work sustain the idea that during lineage reprogramming cells "hop over a mountain" into the next "valley", using the imagery of the epigenetic landscape introduced by Waddington (Waddington, 1957). The finding that lineage reprogramming does not involve a retrodifferentiation to a pluripotent state might avoid the generation of cell with tumorigenic

potential after transplantation, a key complication of induced pluripotent stem cells approaches in regenerative medicine. This offers a potential strategy for generating cells desired for cell therapy.

2. Cell cycle-dependency

At the beginning of the work about the cell cycle-dependency it was unclear whether reprogramming of pre-B cells to macrophages can occur without cell division or requires one or more cell divisions (Fig. 6).

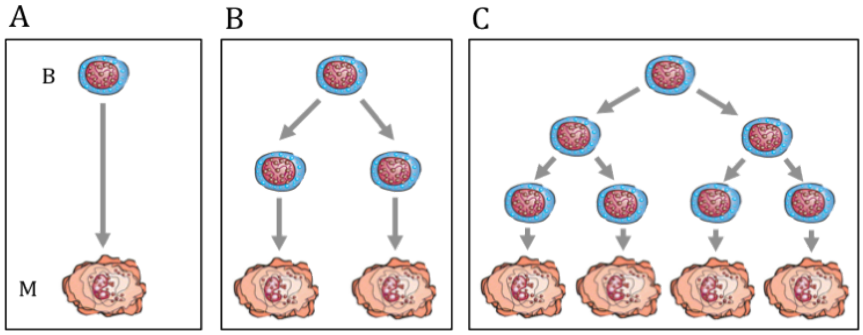


Figure 6. Scenarios of cell divisions during reprogramming. A, direct conversion of B cells (B) to macrophages (M) occurs without cell proliferation; B, one cell division is required before transdifferentiation; C, two (or more) cell divisions are required.

It is generally thought that epigenetic changes that underlie reprogramming events are most easily made during cell division (Hochedlinger and Jaenisch, 2006). It may be the case that many reprogramming events do indeed involve obligatory proliferation steps (Slack, 2007). In contrast, reprogramming of pancreatic endocrine cells to β-cells and of fibroblasts to neurons seem to be cell cycle independent (Zhou et al., 2008) (Vierbuchen et al.) YEAR Early SCNT experiments also provide evidence for reprogramming without DNA replication (De Robertis and Gurdon, 1977). Our results have likewise shown that cell division is not essential for transdifferentiation although strikingly we most cells do divide. Thus, time-lapse experiments showed that the small subset of non-dividing cells corresponds to the most rapidly transdifferentiating cells and that these also express the highest levels of

C/EBPα. In addition, high levels of C/EBPα both accelerate transdifferentiation and increase the proportion of non-dividing cells that turn into macrophages. This dosage-dependent effect is specific for C/EBPα-induced reprogramming since the transdifferentiation driven by C/EBPβ, a transcription factor that has no cell cycle inhibitory activity, is accompanied by cell divisn in 100% of the cells. It has been reported that dephosphorylation of C/EBPα at Ser193 blocks its cell cycle inhibitory activity and promotes cell proliferation (Wang and Timchenko, 2005). This would predict that reprogramming pre-B cells with this dephosphorylated form of C/EBPα all the cells would cycle during transdifferentiation.

The observations that the DNA synthesis inhibitor aphidicolin impaired Mac-1 upregulation but that the S phase itself does not represent a checkpoint required for myeloid gene activation, as indicated by our cell synchronization experiments, is an apparent discrepancy. A possible explanation is that when C/EBPα induced cells are prevented from cycling they accumulate a putative inhibitor of a myeloid effector or perhaps C/EBPα itself. Alternatively, arrested cells may not be able to accumulate sufficient levels of a hypothetical co-factor required for myeloid differentiation. Moreover, knocking down p53 in our pre-B cell system did not alter the transdifferentiation kinetics, thus differing from iPS reprogramming of somatic cells (Hanna et al., 2009). The fact that the C/EBPα induced cells are not prevented by shp53 from arresting in G0 suggests that the cell cycle inhibitory effect of C/EBPα is dominant over the accelerating effect of shp53. Whether this plays out at the level of transcriptional regulation of the cell cycle inhibitor p21, a direct target of both C/EBPα and p53 (el-Deiry, 1998; Timchenko et al., 1996), remains to be determined.

Our observations illustrate an important difference between transcription factor induced transdifferentiation and iPS cell reprogramming, lending

further support to the postulate (Graf and Enver, 2009) that the two processes are fundamentally different.

The example of direct reprogramming described above togheter with the other reprogramming systems, may very well also become important tools for both basic biology and regenerative medicine. These applications fall into 3 principal categories: (i) Utilization of induced cell types to study basic mechanisms of transcription factor action, chromatin remodeling processes, and lineage determination; (ii) Efficient access to human tissue not otherwise accessible for drug testing and disease modeling; (iii) Use of induced cell types for therapeutic cell transplantation. In principle these applications are very similar to iPS cell-based approaches which have been reviewed extensively before (Stadtfeld and Hochedlinger) (Saha and Jaenisch, 2009) but certain advantages and disadvantages compared to direct cell type induction do exist that are often specific to the respective cell type.

Along with differentiation of specific cell types from pluripotent stem cells, direct lineage conversion provides a simplified tool for studying developmental processes *in vitro* (Zhang et al.). These accessible culture systems can be used to search for novel cell fate determinants by candidate gene approaches and with unbiased genomic screens. While iPS cell differentiation is preferable for studying early developmental processes (such as neural induction), the strength of direct lineage reprogramming approach may lie in studying terminal differentiation and maturation, as well as the acquisition of functional properties, processes which are relatively poorly understood. The combination of these two approaches provides a powerful toolkit for studying the development of a variety of cell types *in vitro*.

Perhaps the most exciting future application of these novel "tools" is the possibility of increased experimental accessibility to human cell types in culture. However, only a few examples of direct reprogramming of human somatic cells have been reported, although many are likely forthcoming in

the near future (Szabo et al.) (Pang et al.). While iPS cells have the clear advantage of unlimited growth, thus making them amenable for use in high-throughput assays, it is technically very challenging to produce iPS cell lines from numerous individuals. Unless a proliferative intermediate can be induced, direct lineage converted cells would also need to be scaled up as before conversion. However, since reprogramming is fast and efficient the screening of dozens or even hundreds of individuals may become feasible as methods for lineage reprogramming improve.

Finally, induced lineage reprogramming could be used for autologous therapeutic cell transplantation. iPS cells offer the advantage of scalability but are known for their ability to form teratomas when not properly differentiated. Directly induced lineage reprogramming on the other hand would bypass the pluripotent state and thus would presumably be less tumorigenic, provided integration-free gene delivery methods are applied (Angel and Yanik) (Warren et al.). Another interesting potential application of direct lineag reprogramming would be the use of the reprogramming factors directly *in vivo* e.g. (Zhou and Melton, 2008). Although limited by the well-known complications associated with *in vivo* gene delivery, this approach would eliminate the lengthy process of culturing explanted cells for lineage conversion. Such approaches may be interesting to explore for myocardial infarction, diabetes and neurodegenerative disorders such as Parkinson's disease.

# Conclusions

No retrodifferentiation

1. Gene expression changes show mostly a direct conversion of pre-B cells into macrophages;
2. Comparison with hematopoietic precursor transcriptomes revealed a subset of transiently activated myelo-monocytic precursor genes;
3. Low levels of *Kit* and *Flt3* mRNAs became upregulated in a developmentally regulated fashion;
4. Cell surface antigens that define multipotent hematopoietic program did not become re-expressed, not even intracellularly;
5. Time-limited activation of C/EBPα fails to induce progenitor antigen expression;

Cell cycle-dependency

6. C/EBPα blocks the cell cycle one day after the induction of reprogramming specifically in G0/G1;
7. BrdU analysis showed that a subset of pre-B cells induced by C/EBPα transdifferentiate without exhibiting DNA synthesis;
8. Inhibition of cell division with aphidicolin impairs the establishment of the myeloid program;
9. The cell cycle stage of pre-B cells does not influence the reprogramming kinetics;
10. Time-lapse experiments reveal a subset of rapidly transdifferentiating cells not requiring cell division.
11. Pre-B cells that transdifferentiate without cell division express the highest C/EBPα levels

# ANNEX 1

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# ANNEX 2

ABBREVIATIONS

| | |
|---|---|
| bZIP | Basic-region leucine zipper |
| b-Est | b-Estradiol |
| BrdU | Bromodeoxyuridine |
| CLPs | Common lymphoid progenitors |
| CSFE | Carboxyfluorescein Diacetate, Succinimidyl Ester |
| DAPI | 4',6-diamidino-2-phenylindole |
| Dox | Doxycycline |
| EPO | Erythropoietin |
| ER | Estrogen Receptor |
| ES | Embryonic stem cells |
| EpiSCs | Epiblast stem cells |
| FLT3 | FMS-like tyrosine kinase 3 |
| FSC | Forward scatter |
| GFP | Green fluorescent protein |
| GMPs | Granulocyte monocyte progenitors |
| hCD4 | Human cluster of differentiation 4 |
| HPCs | Hematopoietic precursor cells |
| HSPCs | Hematopoietic stem and progenitor cells |
| IL3 | Interleukin-3 |
| IL7 | Interleukin-7 |
| iPS | Induced pluripotent stem cells |
| LMPPs | Lymphoid-primed multipotencial progenitors |
| LT-HSC | Long-term hematopoietic stem cells |
| M-CSF | Macrophage colony stimulating factor |
| Mac | Macrophages |
| maGSCs | Multipotent adult germ-line stem cells |
| MAPCs | Multipotent adult progenitor cells |

| | |
|---|---|
| MASC | Multipotent adult spermatogonial stem cells |
| MEFs | Mouse embryonic fibroblasts |
| MkP | Megakaryocyte precursors |
| OSKM | Oct4/Sox2/Klf4/c-Myc |
| PBS | Phosphate buffered saline |
| PGCs | Primordial germ cells |
| preCFUE | Erythroid precursors |
| preGMP | Granulocyte monocyte precursors |
| preMegE | Megakaryocyte erythroid precursors |
| SCF | Stem cell factor |
| SCNT | Somatic cell nuclear transfer |
| SSC | Side scatter |
| ST-HSC | Short-term hematopoietic stem cells |
| TPO | Thrombopoietin |
| USSCs | Unrestricted somatic stem cells |

# ANNEX 3

PUBLICATIONS

1. Rodríguez-Ubreva J, Ciudad L, Gómez-Cabrero D, Parra M, Bussmann LH, **Di Tullio A**, Kallin EM, Tegnér J, Graf T, Ballestar E.
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3. Varas F, Stadtfeld M, de Andres-Aguayo L, Maherali N, **Di Tullio A**, Pantano L, Notredame C, Hochedlinger K, Graf T.
Fibroblast-derived induced pluripotent stem cells show no common retroviral vector insertions.
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4. Marchionni C, Bonsi L, Alviano F, Lanzoni G, **Di Tullio A**, Costa R, Montanari M, Tazzari PL, Ricci F, Pasquinelli G, Orrico C, Grossi A, Prati C, Bagnara GP.
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