Induced transdifferentiation of human B-leukemia/lymphoma cell lines and inhibition of leukemogenicity

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To my family

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THESIS ABSTRACT

B-cell malignancies encompass a wide variety of distinct diseases including Non Hodgkin lymphoma (NHL) and leukemia. Currently, chemotherapy, radiation and anti-CD20 antibody treatment are the mainstays of B-cell lymphoma and leukemia therapy. However, the fact that a large number of patients are eventually not cured justifies the search for novel and more effective therapeutic approaches. Although induction of differentiation has been shown to be effective in several tumors such as acute promyelocytic leukemia, it has not been tested yet in NHL and leukemia. We therefore hypothesized that transdifferentiation of malignant B cells could be proposed as a novel therapeutic approach. Earlier work of our laboratory demonstrated that the transcription factor C/EBPα could convert immature and mature murine B lineage cells into functional macrophages at high efficiencies. Here we show that the ectopic expression of C/EBPα can likewise induce the conversion of selected human lymphoma and leukemia B-cell lines into macrophages. The reprogrammed cells are functional and quiescent. Importantly, the tumorigenicity of transdifferentiated lymphoma and leukemia cell lines was impaired after transplantation into immunodeficient mice, even when C/EBPα was activated *in vivo*. In summary, our experiments show for the first time that human cancer cells can be induced to transdifferentiate by C/EBPα into seemingly normal cells at high frequencies, thus proposing transdifferentiation as novel therapeutic approach. In line with this, we believe that the finding of a small molecule that mimics C/EBPα overexpression will

open new horizons for the cure of patients affected by B cell malignancies.

RESUMEN DE TESIS

Las neoplasias malignas de células B abarcan una amplia variedad de enfermedades diferentes, incluyendo el linfoma no Hodgkin (LNH) y leucemia. Actualmente, la quimioterapia, la radiación y el tratamiento con anticuerpos anti-CD20 son los pilares de la terapia contra el linfoma y la leucemia de células B. Sin embargo, el hecho de que un gran porcentaje de pacientes no se cura con estos tratamientos, justifica la búsqueda de nuevas terapias más eficaces. Aunque la inducción de la diferenciación ha demostrado ser eficaz en el tratamiento de varios tumores tales como la leucemia promielocítica aguda, esta técnica no se ha probado aún en el tratamiento del LNH o de la leucemia. Por lo tanto, la transdiferenciación de las células B malignas podría ser propuesta como un nuevo enfoque terapéutico. Trabajos anteriores de nuestro laboratorio han demostrado que el factor de transcripción C/EBPα puede convertir células de linaje B murinas inmaduras y maduras en macrófagos funcionales con una alta eficiencia. En este trabajo mostramos que la expresión ectópica de C/EBPα puede inducir la conversión de ciertas líneas de linfoma y leucemia humana en macrófagos. Las células reprogramadas son funcionales y quiescientes. Es importante destacar que la tumorigenicidad de linfoma transdiferenciados y líneas celulares de leucemia se vio afectada después del trasplante en ratones inmunodeficientes, incluso cuando C/EBPα se activó *in vivo*. En resumen, nuestros experimentos muestran por primera vez que las células de cáncer humano pueden ser inducidas por C/EBPα a transdifferenciarse en células aparentemente normales con una alta frecuencia,

proponiendo así la transdiferenciación como nuevo enfoque terapéutico. En línea con esto, creemos que el hallazgo de una pequeña molécula que sea capaz de imitar la sobreexpresión de C/EBPα abrirá nuevos horizontes para la cura de los pacientes afectados por tumores malignos de células B.

PREFACE

For decades cell fate decisions were thought to be a unidirectional and irreversible process, and fully differentiated cells were thought not to have any plasticity.

However, studies performed using different techniques such as nuclear transfer, cell fusion and transcription factor transduction, demonstrated that fully differentiated cells are plastic and indeed is possible to force them to acquire new fate. Cell fate decisions can be altered by converting a somatic cell back into a pluripotent state (reprogramming), or by directly converting one differentiated cell type into another, without passing through a pluripotent state (transdifferentation). So far many examples of inter and intra germ layer transdifferentiation have been reported, using single or combination of transcription factors, but none of these studies assessed if it is possible to directly convert a malignant cell type into a “normal state’. Moreover, both reprogramming and transdifferentiation efficiency, have been reported to be higher in mouse cells compared to human ones. Our experiments show that human malignant B-cell lines can be induced to transdifferentiate by C/EBPα into seemingly normal cells at high frequencies, providing a proof of principle for a potential new therapeutic strategy to treat B cell lymphoma and leukemia.

PART I

INTRODUCTION AND AIMS

Introduction

1. DEVELOPMENT AND CELL PLASTICITY

In mammals, embryo development is a unidirectional process in which cells become progressively restricted in their potency (Fig 1). Cells at early stages, such as the zygote and the first cleaved blastomeres, are able to generate the entire organism as well as extra-embryonic tissues, forming the so-called *totipotent* cells.

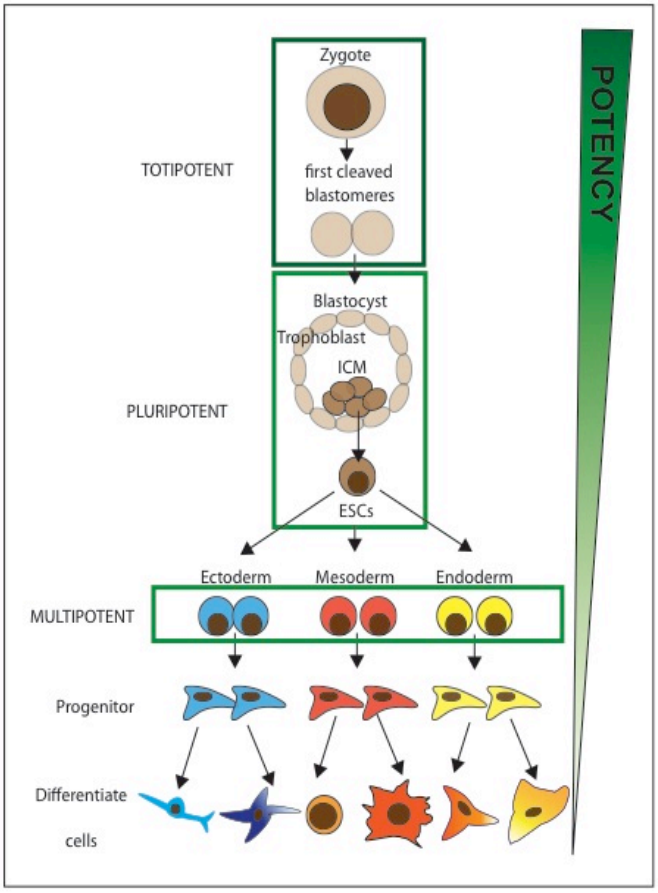

Fig.1 Scheme representing normal differentiation and lost of potency during development: from totipotent cells to plutipotent and bi-or monopotent progenitor.

During later stages, the blastocyst becomes composed by two lineages: the trophoblast, which will form part of the placenta,

and the inner cell mass (ICM) that will give rise to all the different cell types of the organism. These two cell types don't have the ability to interconvert and are restricted in their development potential (Jaenisch and Young, 2008). At the blastocyst stage, embryonic stem cells (ESCs) can be derived from the ICM (Evans and Kaufman, 1981; Martin, 1981). ESCs are termed *pluripotent* cells since they retain the capability of the ICM to differentiate in all the three germ layers -the ectoderm, the endoderm and the mesoderm- *in vivo* and *in vitro* under proper culture conditions (Boiani and Scholer, 2005). ESCs differentiate into *multipotent* cells able to generate a limited number of cell types belonging to the same lineage. One example of multipotent cells are hematopoietic stem cells (HSCs) that can give rise to all the fully differentiated cell type of the blood system (Orkin and Zon, 2008).

For many years Waddington's epigenetic landscape model (Fig.2a) has been used as a metaphor for biological development (Waddington, 1957). In the revisited model from Graf and Enver, pluripotent stem cells are represented as a marble at the top of a mountain (Graf and Enver, 2009). When they roll over the ridge of the mountain they follow different valleys on the slope and become progressively restricted in their developmental potential. Finally when the cells reach the bottom of the mountain they acquire their fully differentiated and stable state. For decades cell fate decisions were thought to be a unidirectional and irreversible process, and fully differentiated cells were thought not to have any plasticity.

Nevertheless, some classical studies suggested that committed cells of the embryo are susceptible to cell fate changes, putting forward the new concept that also differentiated cells may retain

some plasticity. One of the studies that demonstrated cell plasticity in progenitors was done by Hadorn and colleagues in 1968. They showed that in *Drosophila*, single cells from the imaginal discs - primitive larval structures that will form different appendages in the adult - can change their fate if serially transplanted to ectopic sites in the larva. Indeed cells isolated from the wing disc were shown to be able to form legs (Hadorn, 1968). In another study, Le Lievre and Le Douarin proved that explanted neural crest cells from quails transplanted into chickens adopted new fates (bone, cartilage and connective tissue) dictated by their new cellular environment (Le Lievre and Le Douarin, 1975). This phenomenon, termed transdetermination, highlighted the fact that committed progenitor cells can switch their fate under appropriated environmental stimuli, advancing the concept that also more differentiated cells retain some plasticity.

2. STATEGY FOR CELL FATE CONVERSION AND REPROGRAMMING

Waddington's model (Fig.2a) describes the natural restriction of cell differentiation potential during normal development. However, in the last decades, several experiments have been performed to demonstrate that fully differentiated cells are indeed plastic and that is possible to force cells fate decisions. To this end, different techniques such as nuclear transfer, cell fusion and transcription factor transduction have been used (reviewed in (Graf, 2011; Yamanaka and Blau, 2010). Cell fate decisions can be altered by converting a somatic cell back into a pluripotent cell (Fig.2b) (reprogramming), or by directly converting one differentiated cell type into another, without passing through a pluripotent state (Fig.2c) (also called lineage conversion or transdifferentiation) (Ladewig et al., 2013).

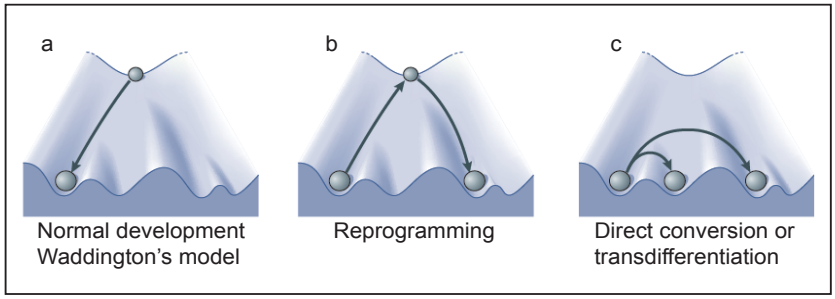

Fig.2 New view of the Waddington's epigenetic landscape model taking into account somatic cell plasticity (adapted from Ladewig et al., 2013).

2.1 REPROGRAMMING BY SOMATIC CELL NUCLEAR TRANSFER

A key discovery in the understanding of cell differentiation was the development of the somatic cell nuclear transfer (SCNT) technique (Briggs and King, 1952). This advance was fundamental for Kings and Briggs to discover that the transfer of nuclei of frog cells (Rana pipiens) isolated from early blastocysts into enucleated oocytes resulted in the formation of swimming tadpoles. The authors also showed that nuclei isolated from more "differentiated" cells from the gastrula stage on failed in forming tadpoles when transplanted, suggesting that more advanced differentiation stage lead to a loss of cell plasticity (King and Briggs, 1955). On the contrary, using another species of frogs, *Xenopus laevis*, the studies of John Gurdon demonstrated that mature fertile frogs could be obtained through transfers of nuclei from cultured intestinal cells of tadpoles (Gurdon et al., 1958; Gurdon and Uehlinger, 1966). Even though the incidence of nuclear reprogramming was very low (around 1%), Gurdon's cloning experiments demonstrated for the first time that mature cells retain in the nucleus all the genetic information of a pluripotent cell and that differentiation is a reversible process. A long time passed before the cloning of the first mammal was achieved. In 1997 Wilmut and colleagues were able to clone a sheep, (*Dolly)*, after the transplantation of nuclei from cultured epithelial cells into enucleated oocytes (Wilmut et al., 1997). A year later Wakayama and colleagues succeeded in cloning the first mice (Wakayama et al., 1998). However, despite all the experimental proofs provided by the authors, the doubt

that the cloned animals obtained so far could have arisen from contaminating stem cells or multipotent progenitors persisted in the scientific community. The final proof, that specialized cells could be used for the cloning of an organism, was provided by work of the Jaenisch laboratory. They unequivocally demonstrated that the nucleus of a mature lymphocyte, containing immunoglobulin heavy chain (IgH) rearrangement, could be reverted to produce a mouse clone carrying fully rearranged immunoglobulin alleles in all tissues (Hochedlinger and Jaenisch, 2002). In addition to sheep and mice, a wide range of species have been cloned using the SCNT, from domestic to wild animals (Wakayama and Wakayama, 2010). However, the efficiency of cloning remains very low (1-2%); moreover the cloning efficiency varies a lot depending on the cell type and the developmental stage of the nuclear donor. Notably, nuclear-transfer-derived ES cells (ntES cells) can be generated with much higher efficiency (20%) from blastocysts formed by SCNT (Yang et al., 2007). After the generation of nt-ES cells from non–human primates (Byrne et al., 2007), very recently the same group have shown the reprogramming of human fibroblasts into nt-ES cells using an optimized SCNT protocol, opening a new avenue for human therapeutic cloning. (Tachibana et al., 2013). Interestingly, cloned animals often show abnormalities such as aberrant gene expression in the embryo, telomerase elongation, obesity in adults, impaired immune system and often increased cancer susceptibility and premature death (Thuan et al., 2010). The low efficiency of the cloning process and the abnormalities developed in the cloned animals suggests that the reprogramming is incomplete, most probably due to failure in erasing ‘epigenetic memory’, defined

as effects on gene expression not due to differences in DNA sequences (Simonsson and Gurdon, 2004).
However the generation of nt-ESCs and animals by SCNT demonstrated that the epigenetic state of a differentiate somatic cell is not fixed but can be reprogrammed to an embryonic state capable of differentiate in different cell types and eventually generate a new organism.

2.2 CELL FUSION

Cell fusion allows the generation of a single cellular entity by fusing one or more cell types. Upon cell fusion, two different hybrids can be formed:
- synkaryon: proliferating hybrids containing a single nucleus
- heterokaryon: non- proliferating hybrids containing two nuclei

The advantage of using cell fusion to study early phases of reprogramming is due to the possibility to use non-dividing heterokaryons, avoiding in this way, chromosome loss, rearrangements and aneuploidy. Moreover, the use of mixed species heterokaryons allow the study of early molecular events during reprogramming, such as gene expression changes that can be monitored on the basis of species-specific differences at the genome or single cell level (Soza-Ried and Fisher, 2012).
The first indication that reprogramming could be achieved by cell fusion came from the studies performed by Miller and Ruddle. They demonstrated that heterokaryons formed by the fusion of embryonal carcinoma cells with thymus cells generate the formation of teratocarcinomas containing various differentiated tissues when injected into mice, (Miller and Ruddle, 1976). The

evidence that previously silenced genes can be reactivated in fused cells, come from experiments performed in Blau's laboratory. Using heterokaryons formed from the fusion of mouse muscle cells and normal diploid human amniocytes the authors were able to show the re-activation of human muscle genes. Moreover, they also observed the expression of several human muscle proteins in non-muscle cells together with direct differentiation (Blau et al., 1983). Afterwards, mouse muscles cells were fused with diverse cell types, including human fibroblasts (mesoderm), hepatocytes (endoderm) and keratinocytes (ectoderm), demonstrating that expression of muscle specific genes could be activated in cells from the three different germ layers (Blau et al., 1985). Some years later, Tada and colleagues were able to show reprogramming to pluripotency of somatic cells by cell fusion. They fused embryonic germ cells (pluripotent stem cells) from a female mice with thymocytes from adult male mice and showed epigenetic changes such as demethylation and re-activation of non-imprinted genes in the somatic nucleus. Moreover, the ES-thymocyte hybrids contribute to all three germ layer of chimeric embryos (Tada et al., 1997). The same authors showed that hybrids formed between female thymocytes and male ESCs re-activate the pluripotent gene Oct-4 and the silenced X chromosome. (Tada et al., 2001). These studies were subsequently extended by Cowan and colleagues that succeeded in reprogramming human fibroblasts by fusing them with human ES cells (Cowan et al., 2005). Other studies performed by fusing somatic cells with pluripotent cells have elucidated the mechanisms underlying reprogramming. For instance, assays in which ESCs were fused with neuronal stem

cells, fibroblasts or thymocytes demonstrated that the pluripotency gene, Nanog, can promote reprogramming (Silva et al., 2006). Further studies have shown that fusion of mouse ESCs cells with human lymphocytes initiate the expression of human stem cell transcription program (Pereira et al., 2008). Cosma's lab and others have shown that activation of the Wnt/β-catenin pathway increases reprogramming efficiency by overcoming Tcf3-mediated repression of pluripotency genes (Lluis et al., 2011; Lluis et al., 2008; Marson et al., 2008; Ombrato et al., 2012). Recently it has been shown that DNA synthesis is required for reprogramming mediated by stem cell fusion (Tsubouchi et al., 2013).

The studies using cell fusion showed that one cell state is dominant over the other (especially that of pluripotent cells) and that expression of previously silenced genes can be reactivated through the modulation of epigenetic markers. Moreover they showed that is possible to reprogram the nucleus of a somatic cell back to pluripotency, reinforcing the concept that fully differentiated cells are indeed plastic.

2.3 KEY TRANSCRIPTION FACTORS TRANSDUCTION EXPERIMENTS

One of the first reports of conversion of cell fate to another using overexpression experiments was performed in 1987. Gehring and colleagues showed that the overexpression of a homeotic gene, Antennapedia, in *Drosophila melanogaster* larvae led to changes in body plans, giving rise to legs instead of antennae (Schneuwly et al., 1987). A decade later, even more striking was

the discovery made by the same group that ectopic expression of eyeless (Pax6 in mammals) in *Drosophila* led to development of functional eyes on legs, wings and antennas of the fly (Gehring, 1996). However, these *in vivo* experiments could also be interpreted as re-specification of progenitors cells and moreover do not clarify if master regulators can act only on progenitors cells or are also capable of promoting direct somatic cell fate conversion. Nevertheless they show that cells are not stably fixed in their differentiation stage. The discovery of MyoD as a tissue-specific master regulator transcription factor, and the following demonstration that overexpression of MyoD induces phenotypic conversion of fibroblast into muscle cells (Davis et al., 1987)(Davis et al., 1987; Taylor and Jones, 1979), was the first example of transdifferentiation in the mammal system. Subsequent experiments showed that MyoD can also convert pigment, nerve, fat and liver cell lines into cells that express muscle markers, however the resulting cells looked aberrant and were generated only a very low efficiency (Weintraub et al., 1989). Subsequently, Graf and colleagues have shown that ectopic expression of GATA-1 in myeloblasts convert them into megakaryocytes-erythrocytes precursors suggesting that transcription factors can not only activate genes specific of a particular lineage but at the same time are able to repress the gene expression program specific of the starting cell, an hallmark of transdifferentiation (Graf et al., 1992). Moreover, our group have shown that fully differentiated primary B cells can be converted into functional macrophages at high efficiency by the overexpression of a single transcription factor: C/EBPα (Xie et al., 2004).

Afterwards years various studies have extensively shown the direct cell fate conversion of virtually any differentiated cell type into another one. Transdifferentiation studies will be discussed in details in the next paragraph.

A breakthrough in the reprogramming field, has been made in 2006 by Takahashi and Yamanaka. They showed that the overexpression of the transcription factors Oct4, Sox2, Klf4 and Myc in mouse fibroblasts is able to reprogram them into an ES-like pluripotent state, Therefore these cells were called induced pluripotent stem cells (iPS cells). Pluripotency of the generated cells was assessed by capacity for multilineage differentiation as well as activation of endogenous pluripotent factors expressed in ESCs, such as Oct-4 and Nanog. Supporting the *in vitro* findings, injection of the reprogrammed cells into mice led to the formation of teratomas (tumors containing the three germ layers (Takahashi and Yamanaka, 2006). One year later the same group was able to reprogram human fibroblasts into iPS cells using the same cocktail of factors (Takahashi et al., 2007), together with another group that used a different combination of factors (Oct-4, Sox-2, Nanog and Lin-28) (Yu et al., 2007) . Since then, many scientist were able to reproduce these findings and generated iPS cells from different types of fully differentiated cells, such as B cells (Hanna et al., 2008) and post-mitotic neurons (Kim et al., 2011).

Transdifferentiation and reprogramming experiments showed unambiguously that somatic cells contain in their nucleus all the information of differentiation potential and by perturbing the regulatory networks that control the stability of a differentiated cell it is possible to convert a committed cell into another differentiated state or even back to pluripotency. Waddington's

model is still useful to explain normal tissue development, but recently a new model was proposed to explain somatic cell plasticity that lacks hierarchy between cell types (Fig.3). In this flat disc model cells are represented at the edges of the disc and the pluripotent state is just one of many possible states, symbolized as an open hutch. In this model, cells can be moved from one state to another by exogenous factors, that tilt the disc in a specific direction (Ladewig et al., 2013).

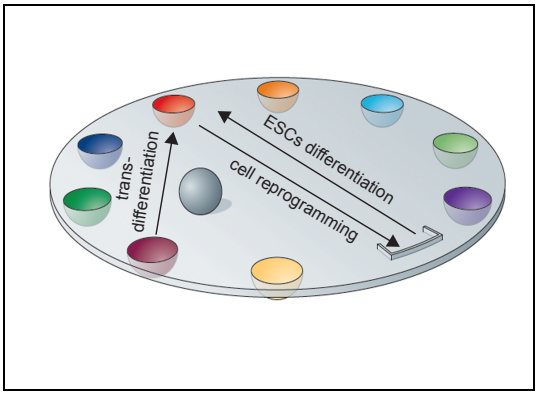

Fig.3 The epigenetic flat disc model (adapted from Ladewig et al., 2013).

3. DIRECT CELL FATE CONVERSION

The conversion of fibroblasts into muscle cells and the twotransdifferentiation examples within the hematopoietic system discussed in paragraph 1.3, opened new horizons in developing strategies of cell fate conversion guided by master regulators. Indeed, various studies have shown that by forced expression of cell lineage-instructive transcription factors a specific cell type can be converted into a different cell type belonging to the same germ layer (intra-germ layer) or to a different germ layer (inter-germ layer).

3.1 INTRA GERM LAYER CONVERSION

3.1.1 Mesoderm

This section focuses on cell fate conversions within the hematopoietic system (Fig. 4) and other transdifferentiation systems are only briefly discussed.

Adult hematopoietic stem cells (HSCs) reside in the bone marrow of adult mammals and are able to both self-renew and differentiate into progenitors restricted in their differentiation potential. HSCs give rise to common myeloid progenitors (CMPs) that further differentiate into granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythroyd progenitors (MEPs). In addition, HSCs differentiate into common lymphoid precursors (CLPs). More in detail, GMPs differentiate into the myeloid lineage (mast cells, eosinophils, granulocytes/neutrophils and monocytes/macrophages) whereas MEPs generate erythrocytes and megakaryocytes. On the other hand, CLPs give rise to the lymphoid lineage (mature B, T and natural killer (NK) cells) (Orkin and Zon, 2008). The transcription factors governing lineage differentiation within the hematopoietic system are well studied (reviewed in (Graf and Enver, 2009; Orkin and Zon, 2008). The transcription factors GATA-1 and PU.1 control CMP differentiation into MEPs and GMPs, respectively (Arinobu et al., 2007; Iwasaki and Akashi, 2007). Lineage conversion experiments showed that forced overexpression of PU.1 is sufficient to convert MEPs into myeloblasts (Nerlov and Graf, 1998). On the other hand, overexpression of GATA-1 in monocytic cell lines is sufficient to downregulate specific monocyte markers and promote the

expression of eosinophil, erythroid and megakaryocytic markers (Kulessa et al., 1995). Moreover GATA-1 expression into GMPs and CLPs promotes differentiation into erythroid, eosinophil, basophil and megakaryocytic lineages (Iwasaki and Akashi, 2007).

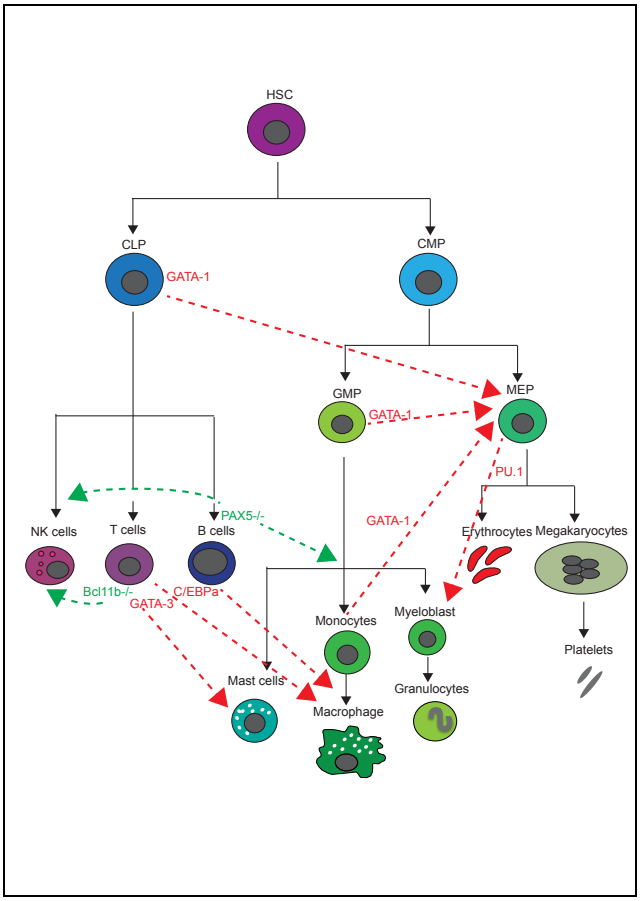

Fig.4 Transdiferentiation in the hematopoietic system. The red dashed arrows depict direct cell fate conversions upon overexpression of the transcription factors PU.1, GATA-1, GATA-3 and C/EBPα. Green dashed arrows indicate cell fate conversion performed with ablation of a transcription factor.

Another example of cell fate conversions within the hematopoietic system is that of fully differentiated B and progenitors T cells into functional macrophages by the

overexpression of the transcription factor CAAAT/enhancer binding protein alpha (C/EBPα) (Xie et al., 2004) (Laiosa et al., 2006). The direct conversion of fetal thymocytes into mast cells has been achieved by the overexpression of the GATA-3 transcription factor (Taghon et al., 2007). Interestingly, also master regulator ablation has been reported to have a role in cell fate specification changes. For instance, Pax5-/- pro B cells can differentiate into functional macrophages, osteoclasts, dendritic cells, granulocytes and natural killer cells under proper culture conditions (Nutt and Kee, 2007). Moreover, deletion of Bcl11b is sufficient to convert T cells into functional NK cells (Li et al., 2010a; Li et al., 2010b). A very recent paper reports the conversion of mouse fibroblasts into hemogenic progenitors opening the avenue for the potential generation and expansion of HSCs *in vitro* (Pereira et al., 2013).

Beside the hematopoietic system, another transdifferentiation within the mesoderm has been reported. Kajimura and colleagues have shown that expression of PRMD16 and C/EBPβ is sufficient to induce a fully functional brown fat program in mouse and human fibroblasts (Kajimura et al., 2009).

Although all the examples of cell-fate conversion described above elucidated novel regulatory mechanisms underlying the control of the hematopoietic and brown fat systems, so far none of these have been reported to have a therapeutic application. On the contrary the forced cell fate conversion of fibroblasts into cardiomyocytes is promising for its application in cell therapy. Overexpression of the three transcription factors GATA-4, MEF2C and t-box (TBX5) (GMT) is sufficient to convert dermal fibroblasts into cardiomyocyte-like cells (Ieda et al., 2010) *in vitro* and *in vivo*. Moreover, transdifferentiated cardiomyocytes are

able to ameliorate cardiac dysfunction in mice (Qian et al., 2012). In addition to transcription factors also microRNAs (miRNAs) can mediate cell fate conversions. For instance, the expression of four miRNAs involved in heart development (miR-1,miR-133, miR-208 and miR-499) can convert mouse fibroblasts into cardiomyocytes *in vitro* and *in vivo* (Qian et al., 2012).

3.1.2 Endoderm

The pancreas and liver arise from adjacent areas in the anterior endoderm of the developing embryo (Wells and Melton, 1999), and have been proposed to come from common progenitor cells (Deutsch et al., 2001). By default the common progenitor cells differentiate into pancreatic cells, but an FGF-like signal released from the cardiac mesoderm diverts the fate of some cells into liver cells (Jung et al., 1999). Moreover, the transdifferentiation of pancreatic into liver cells (and vice versa) seems to be a physiopathological condition since have been observed in animal experiments and in certain human pathologies (Shen et al., 2000). Slack and colleagues have established an *in vitro* system to study the molecular basis of this transdifferentiation and shown that the overexpression of C/EBPβ can convert exocrine pancreas cells into hepatic cells (Shen et al., 2000). The same group has also shown that it is possible to convert liver cells into pancreatic cells *in vivo* (Xenopous) and *in vitro* (human hepatocytes) by the overexpression of the pancreas and duodenum homeobox 1 (PDX1) factor (Horb et al., 2003).

A strong interest in the potential therapeutic applications of transdifferentiation was raised when Melton and colleagues

showed that it is possible to convert exocrine cells into insulin producing beta cells *in vivo* by the over expression of three factors involved in the pancreatic development (PDX1, NGN3, and MafA) (Zhou et al., 2008). This process takes approximately 10 days and has an efficiency of conversion of 20%. Strikingly, these transdifferentiated cells alleviate the hyperglycemia caused by insulin deficiency.

3.1.3 Ectoderm

Also several examples of cell-fate conversion within the ectoderm have been recently reported. For instance early postnatal cortical astroglia in culture can be reprogrammed to adopt a neuronal fate after forced expression of Pax6, a transcription factor (TF) required for proper neuronal specification during embryonic corticogenesis (Heins et al., 2002). Another group, in independent work, has shown that also the proneuronal genes ASCL-1 and NGN2 are able to reprogram astroglial cells from early postnatal cerebral cortex into neurons. However these astroglial derived neurons fail to establish functional synapses (Berninger et al., 2007). Further studies have succeeded in generating functional neurons from post-natal astroglia using a single transcription factor, DLX2. Moreover, the authors demonstrated that distinct neurogenic transcription factors, such as Neurog2 and Dlx2, alone or in combination with Mash1, can indeed instruct the selective generation of different neuronal subtypes, i.e. glutamatergic and GABAergic neurons, respectively (Heinrich et al., 2010; Heinrich et al., 2011). Although these studies provide evidence that inter germ layer

conversion within the ectoderm is possible, they do not assess the possibility to directly convert adult somatic neuronal cells into another cell type. More recently, the direct conversion of human pericyte-derived cells from the adult human cerebral cortex into induced neuronal cells has been shown by co-expression of Sox-2 and Mash1, opening the possibility to translate neuronal reprogramming into therapy (Karow et al., 2012).

3.2 INTRA GERM LAYER CONVERSION

The first example of direct conversion between cells belonging to different germ layers was shown by Wernig and colleagues, who were able to convert mouse fibroblasts into functional neurons by the co-expression of three transcription factors: Ascl-1, BRN2 and MYT1L (Vierbuchen et al., 2010). One year later this approach was used to convert human fibroblasts and endoderm-derived hepatocytes into neurons (Marro et al., 2011; Pang et al., 2011). However, human neurons only become fully reprogrammed by the addition of a fourth factor (NeuroD1). Also miRNAs have been reported to play a role in this transdifferentiation process. Yoo and colleagues, demonstrated that the overexpression of two miRNAs (miR-9/9 and miR-124) alone is able to convert human fibroblasts into neuron-like cells at low frequency. Moreover, co-expression of miRNAs and transcription factors (ASCL-1, MYT1L and NeuroD1) increase the efficiency of conversion and lead to a mature neuronal phenotype (Yoo et al., 2011). Regarding the molecular mechanisms underlying cell-fate conversion, the two miRNAs mentioned have been reported to regulate gene expression by

chromatin remodeling, suggesting that they might function via epigenetic mechanisms (Yoo et al., 2009). Supporting the findings of Yoo et al., Ambasudhan and colleagues have reported that miR-124 together with MYT1L and BRN2 is sufficient to convert human fibroblasts into functional neurons (Ambasudhan et al., 2011). A major concern in the direct conversion into neurons is that these cells are by nature post-mitotic and not amenable for further expansion, thuslimiting their potential clinical application. To bypass this problem, several groups have generated proliferative neuronal progenitors that can be expanded *in vitro* and differentiate into different neuronal types (Han et al., 2012; Lujan et al., 2012; Ring et al., 2012; Thier et al., 2012). Another example of trans-germ layer conversion is the generation of hepatocyte-like cells from mouse fibroblasts. Two independent groups reported this conversion using different combinations of transcription factors. Hepatocyte-like cells generated with the two methods engrafted into the adult liver and were able to slightly increase survival of a genetic mouse model of liver failure. However when comparing the gene expression profile of primary and converted hepatocyte-like cells, significant differences were found (Huang et al., 2011); (Sekiya and Suzuki, 2011).

Together the studies described here highlight not only the key role of master transcription factors to direct fate conversion, but also the importance of epigenetic modifications and microRNAs involved in this process.

4. CCAAT/ENHANCER-BINDING PROTEIN ALPHA

The CCAAT/enhancer-binding protein (C/EBP) family is composed by six members. Each member is designated by a Greek letter, corresponding to the chronological order of their discovery: C/EBP α, β, γ, δ, ε, ζ (McKnight, 2001). All the C/EBP proteins share substantial sequence identity in the C- terminal region, which consists of basic amino-acid-rich DNA binding regions and a leucine zipper dimerization motif (b-Zip domain). Due to the high homology of the leucin zipper domain, C/EBP proteins can form homo- and heterodimers in all intrafamiliar combinations (Ramji and Foka, 2002).

C/EBPα was the first member to be identified as a liver enriched DNA binding protein (Landschulz et al., 1988). Subsequently, it was found to be highly expressed also in adipose tissue, lung epithelium, intestine, placenta and myeloid cells (Ramji and Foka, 2002). In these tissues C/EBPα directly binds to the promoter of lineage specific genes and activates its transcription, contacting the basal transcriptional apparatus (TBP/TFIIB), interacts with histone acetyltransferases (CBP/p300) and recruites chromatin remodelling complexes (SWI/SNF) (Koschmieder et al., 2009). The capacity of C/EBPα to promote differentiation in a tissue specific manner is thought to rest on its ability to collaborate with other transcription factors such as PPARγ in adipocyte differentiation (Tontonoz et al., 1994), FOXA2 in lung epithelium (Cassel et al., 2000) and GATA-1 and PU.1 in the hematopoietic system (McNagny et al., 1998). A

clear role of C/EBPα in tissue development have emerged from the study of C/EBPα knockout animals. These mice die soon after birth of lipodystrophy, lack of granulocytes and lethal perinatal hypoglycemia caused by insufficient hepatic glucose efflux (Wang et al., 1995). Detailed study of the hematopoietic system in C/EBPα -/- mice reveale that the lack of mature granulocytes is due to the lack of granulocyte-monocyte progenitors (GMPs), while the number of common myeloid progenitors (CMP) is normal (Zhang et al., 2004). In fact, conditional deletion of C/EBPα in GMPs allows for normal granulopoiesis *in vitro*, indicating that C/EBPα is not required in granulopoietic differentiation beyond the GMP stage (Zhang et al., 2004). C/EBPα also controls fetal liver HSC self-renewal as C/EBPα -/- HSCs show increased competitive repopulation activity in transplanted mice (Zhang et al., 2004). Very recently, using a conditional knockout mouse, it has been shown that C/EBPα is essential for the acquisition and mantainence of adult HSCs (Ye et al., 2013).

C/EBPα is a particularly interesting transcription factor because it is able to couple two of the main features of terminal differentiation: specifying cell fate and promoting cell cycle exit (Umek et al., 1991). Although C/EPBa is an intronless genes two different proteins isoforms are generated by regulated alternative translation initiation of the same mRNA: a full length protein (p42) and a truncated one (p30) that differs in their the N-terminus domain. The C/EBPα long isoform p42 is able to promote cell cycle arrest through different mechanisms including the interaction with Cdk2, Cdk4 and SWI/SNF chromatin complex, upregulation of p21 and repression of E2F that leads to

c-Myc downregulation (Johnson, 2005; Zhang et al., 2004). Interestingly the short isoform p30 lacks the N-terminus that mediates its antimitotic activity and instead it is able to promote cell differentiation (Calkhoven et al., 2000). For instance, it has been shown that mice lacking only the p42 C/EBPα isoform have GMPs and myeloid progenitors but they fail to fully differentiate and hyperproliferate *in vitro* (Kirstetter et al., 2008).

5. ROLE OF C/EBPα IN HEMATOLOGICAL MALIGNANCIES

Due to its crucial role in myeloid comparment differentiation and in cell cycle arrest, loss-of C/EBPα function results in the aberrant proliferation and accumulation of myeloblasts in the bone marrow of C/EBPα -/- mice. This block in differentiation resembles the phenotype observed in acute myeloid leukemia (AML) in humans (Zhang et al., 2004). In fact, mutations of C/EBPα have been found in acute myeloid leukemia (AML) patients at a frequency of 5-14% (Koschmieder et al., 2009; Lin et al., 2005). The mutations of C/EBPα in AML can be divided in two main groups:

- mutations that affect the N-terminus of C/EBPα, abolish the p42 isoform of C/EBPα, leaving the p30 functional, leading to induction of proliferation;
- mutations that affect the C-terminus of C/EBPα, distrupting the basic zipper region thus affecting its DNA binding capacity as well as its homo and hetero dimerization capacity.

Interestingly, no bi-allellic mutations of C/EBPα have been reported in AML patients, thus some residual C/EBPα activity may be required for the malignat transformation to occur (Fuchs, 2007). This observation correlates well with the observation that C/EBPα -/- mice never become truly leukemic (Zhang et al., 2004). Moreove, the long latency of the development of the disease (10 to 30 years) points towards the possibility that a second genetic hit may be necessary for the development of AML (Fuchs, 2007).

Apart from direct mutations, C/EBPα expression is decreased/abolished in some forms of AML. For instance in AML samples that have the oncogene AML-ETO fusion protein, C/EBPα mRNA is downregulated by a mechanism that is still not completely understood. Conditional expression of C/EBPα in AML-ETO AML is enough to overcome the block in differentiation leading to the production of mature granulocytes (Pabst et al., 2001a; Westendorf et al., 1998). C/EBPα expression has been found to be decreased due to translational regulation in AML samples with the AML-1 MDS1-EVI1 (AME) fusion protein. In these patients the RNA- binding protein calreticuline (CRT) is strongly activated and by binding to the GC-rich stem structure present in the coding region of C/EBPα mRNA inhibits its translation (Helbling et al., 2004). Another example is the inhibition of C/EBPα translation in chronic myeloid leukemia (CML) with BCR-ABL via the RNA binding protein hnRNP-E2 (Perrotti et al., 2002). C/EBPα has been reported to be inhibited also through post-translational modifications. In samples harboring the oncogenic kinase Flt3-ITD, C/EBPα is phosphorylated on serine-21, inhibiting granulocyte

differentiation (Ross et al., 2004). The finding that C/EBPα is often directly mutated in AML or its expression and function altered indicates that C/EBPα can function as a tumor suppressor. The most promising approach for the treatment of AML is the activation of the C/EBPα-differentiation pathway through small molecules. In fact, MAP/ERK kinase (MEK) inhibitors block serine-21 phosphorylation of C/EBPα, increasing the granulocytic differentiation potential (Radomska et al., 2006). Interestingly, FLT3 tyrosine kinase inhibitors also dephosphorylate serine-21 C/EBPα (Radomska et al., 2006). In addition, C/EBPα translation can be efficiently istimulated *in vitro* using the antineoplastic drug 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) which increase the translation of the full-length p42 isoform of the C/EBPα, thereby inducing granulocytic differentiation (Koschmieder et al., 2007).

However, in contrast to its role as a tumor suppressor in AML, a possible role of C/EBPα as an oncogene has emerged from studies on a sub-type of B cell leukemia (acute lymphoblastinc leukemia (ALL)), in which 5 C/EBP family members have been found to be targeted by IGH translocations (Akasaka et al., 2007). In particular, in 6 patients diagnosed with B-ALL having the translocation t(14,19)(q32,q13), a novel molecular breakpoint located most probably in the 3' unstranslated region (UTR) of C/EBPα was found. The C/EBPα mRNA and protein levels in these samples were found to be high, comparable with those of myelomonocytic cell lines, but the samples were negative for all myeloid markers tested, suggesting that no lineage conversion had occurred in these cells (Chapiro et al., 2006).

1. B CELL DEVELOPMENT

B cell development is a multi-step process that starts in the bone marrow generating B cells that have not yet been exposed to external antigens and are called “naïve” or “virgin” B cells. The naïve B cells circulate through the bloodstrem and are delivered to secondary or ‘peripheral’ lympoid organs, in structures called follicles where they undergo antigen-induced differentiation and finally become memory B cells or plasma cells Fig.5 (Kuppers, 2005; Sagaert, 2007; Klein, 2008)

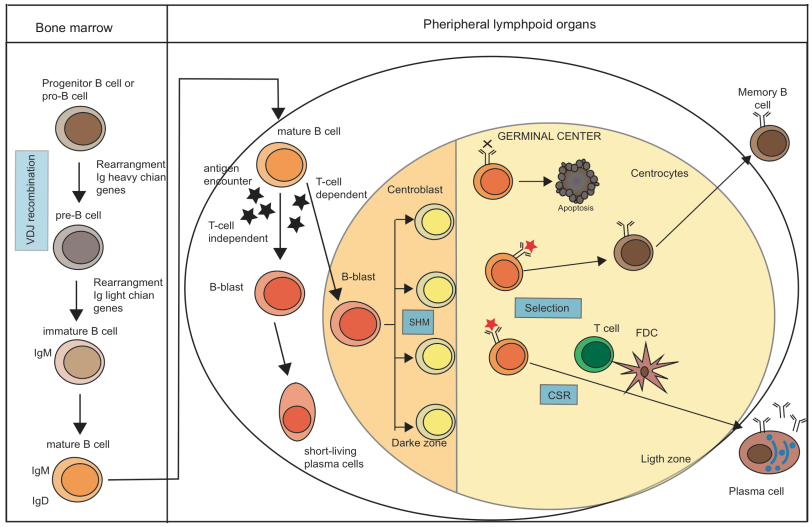

Fig. 5 The differenti steps in B cell developmen, from the bone marrow, though follicles in secondary organs (adapted from (Klein and Dalla-Favera, 2008; Sagaert et al., 2007).

In the bone marrow **progenitor B cells** or **pro-B cells**, that express the B cell antigens Cd19 and CD79a, start to rearrange heavy and light gene immunoglobulin (Ig) genes and this process results in the expression of the B cell receptor (BCR) (Sagaert et al., 2007). Igs are Y-shaped molecules consisting of two heavy (H) and two light (L) chains. The amino-terminal

region of the H and the L chians (V_H and V_L) is called the variable region (V), which confers the ability to bind specific antigens, while the other portion of the H and L chains (C_H and C_L) makes the constant region (C) (Janeway, 2001). To create a functional BCR, the DNA segments that encode the H and L chains must be rearrenged in a process called VDJ recombination (Fig.6a) which occurs within the IGH locus. The heavy chain variable regions are assembled from 123 different variable (V), 27 diversity (D) and 6 junctional (J) genes, wherease the variable region of the light chain (which can be encoded by either the kappa (κ) or the lamda (λ) chain loci) are assembled from V and J elements located at either the Igκ of Igλ gene locus (Sagaert et al., 2007). Once the D_H and J_H genes segments are recombined the **pro-B cell** is transformed into the **early pre-B cell**. **Late pre-B** cells arise when the V_H gene segments is attached to the D_H-J_H segment.

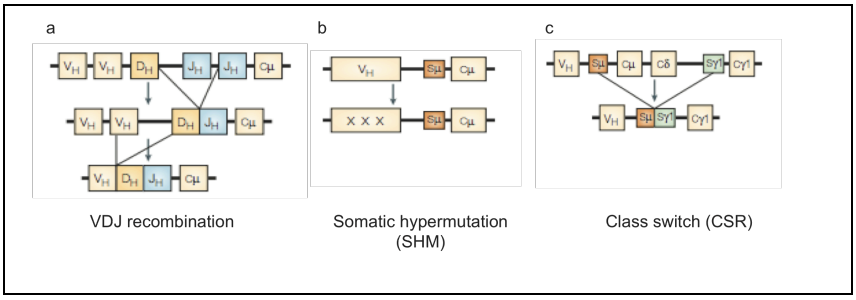

Fig.6 Molecular processes that remodel immunoglobulin genes (adapted from Kuppers, 2005).

Further rearrangments of the light chain locus leads to the expression of IgM molecules and to an **immature B cell** stage. The coexpression of specific classes of immunoglobulins, such

as IgM and IgD, leads to the development of **mature B cells** (Sagaert et al., 2007).

Mature B cells, that express a functional B cell receptor (BCR), leave the bone marrow and migrate into the peripheral lymphoid organs such as spleen, lymph nodes and Peyer's patches where they form primary B cell follicles. Upon antigen encounter, secondary follicles composed of a germinal center (CG) and a sourrounding lymphocytic corona are developed. In spleen and Peyer's patches, where an abundant influx of antigens is known to occur,a distinct zone called marginal zone (MZ) sourrounding the lymphocytic corona can be recognized. The lymphocytic corona and the MZ form the mantle of the B cell follicles (Sagaert et al., 2007).The origin of marginal-zone B cells is debated, and probably includes post-GC memory B cells and naive B cells involved in T-cell-independent immune responses (Kuppers, 2005).

Antigen activated B cells can follow two pathways:

- non GC or extrafollicular path: T cell independent activated mature B cells transform into large B cell blasts that will proliferate in loco giving rise to short living low-affinity antibodies producing plasma cells that are involved in the first line defense against pathogens (primary immune response)
- GC or follicular path: T-cell dependent activated B cells will migrate into germinal centers where they undergo clonal expansion, somatic hypermutation (SHM) and class-switch recombination (CSR), that will produce post-GC high affinity and specialized memory B or plasma cells (secondary immune response) (Sagaert et al., 2007).The GC can be subdivided into dark zone and light zone. In the dark zone are located rapidly dividing large, non-cleaved cells called centroblasts that undergo

somatic hypermutation (SHM) (Fig.6b). The SHM is a process that modifies the Ig variable (V_H) region by introducing non-random mutations, constituted mainly by single nucleotide exchange, but also by small deletions and duplications. B cells that have undergo SHM migrate into the light zone where they are further selected and become small or large cleaved cells called centrcytes (Sagaert et al., 2007). B cells with unfavorable mutations (>90%) that do not bind with high affinity to the antigens presented by follicular dendritic cells (FDCs) or do not interact correctly with T cells undergo apoptosis mediated by the downregulation of the anti-apoptotic gene BCL2. A subset of centrocytes undergo immunoglobulin class switch recombination (CSR)(Fig. 6c) (Sagaert et al., 2007). This process mediates class switches (also called isotype switches), that produce replacement of the originally expressed H chain C- region of the gene with one of another Ig gene, resulting in the production of antibodies with different effector functions but the same antigen binding domain (switch from IgM and IgD to IgG, IgA or IgE). The B cells that produce high affinity antibodies and that have undergone CSR differentiate into memory B cells or plasma cells (antibody secreting cells) (Klein and Dalla-Favera, 2008).

7. HEMATOLOGICAL NEOPLASMS: B CELL LEUKEMIA AND LYMPHOMA

Hematological neoplasm is a general term used to define a broad group of diseases affecting the blood system. Leukemia is a type of cancer characterized by the accumulation of immature cells (blasts). Instead the term lymphoma is used to define blood

cancers that arise when more mature B and T cells are blocked in differentiation. For the purpose of this thesis, classification, key transforming events and their functional consequences will be restricted to B cell acute lymphoblastic leukemias (B-ALLs) and Non-Hodgkin's lymphomas (NHLs).

7.1 LEUKEMIA

Leukemia is described as lymphoid or myeloid, depending on which cell type the malignancy developed from and are further subdivided into:

- Acute leukemia: characterized by a rapid increase of lymphoid progenitors that accumulate in the bone marrow, peripheral blood and occasionally in the central nervous system (Tijchon et al., 2013).
- chronic leukemia: characterized by the accumulation of small B lymphocytes with a mature appearance in blood, bone marrow, lymph nodes, or other lymphoid tissues (Rodriguez-Vicente et al., 2013).

Among the acute lymphoblastic leukemia (ALL) the B cell lineage is more commonly affected that the T lineage (Mullighan, 2012). B-ALL is considered a heterogeneous group of disease because it differs between children and adults and because its molecular pathogenesis, clinical evolution and response to treatment varies widely. B-ALL subtypes have been traditionally correlated with their corresponding B cell developmental stage by immunophenotype, and thus classified accordingly. However this correlation is not always straightforward since there is increasing evidence that several chromosomal transformations found in

childhood B-ALL originate in fetal hematopoiesis in utero. However, the precise origin of the translocations is often difficult to ascertain, especially because the functional consequences of such translocation can appear in further stages of B cell development (Campos-Sanchez et al., 2011). Critical events in the leukemogenesis of B-ALL are recurrent chromosomal rearrangements and aneuploidy. These rearrangements perturb genes encoding regulators of hematopoiesis, tumor suppressors, oncogenes such as tyrosine kinases but commonly require additional genetic hits, such as secondary mutations in the main B cell transcriptional regulators to establish the full leukemic phenotype (Mullighan, 2012). Aneuploidy is an important prognostic factor although its role played in the formation of B-ALLs is poorly understood. In fact, high hyperdiploidy (>50 chromosomes) is associated with a favorable clinical outcome, while aneuploidy (<45 chromosomes) is associated with poor prognosis (Harrison et al., 2004). The most frequent chromosomal translocations that give rise to fusion proteins are: TEL-AML1, MLL fusion proteins, E2A-PBX1 and BCR-ABL. These chimeric proteins have been well characterized. However, the precise mechanisms of how they cause aberrant B cell development are still only partially known. Possible explanations are that chimeric proteins may interfere with the network of B cell regulators or alter the extracellular signaling that regulate normal B cell development in complex ways (Campos-Sanchez et al., 2011).

The TEL-AML1 fusion protein also called ETV6-RUNX-1 is almost exclusively present in childhood ALL (22% vs 2% in adult leukemia). TEL (translocation-ETS leukemia) belongs to the ETS family of transcription factors and is required for the

maintenance of definitive adult HSCs {Hock, 2004 #1799}. Conversely, AML1 (RUNX-1) is essential for the emergence of HSCs in the embryo. The fusion protein TEL-AML1 retains the capacity to bind AML1 targets, but by recruiting a co-repressor complex with histone deacetylases (HDAC) the transcription of its targets genes is repressed, therefore blocking B cell differentiation and promoting self-renewal of B cell progenitors (Hiebert et al., 1996). However the chimeric protein alone is not able to induce leukemia (Andreasson et al., 2001; Morrow et al., 2004). Furthermore, the fusion protein TEL-AML1 is commonly detected at birth (Wiemels et al., 1999), years prior to the onset of leukemia, suggesting that secondary genetic events are required to induce leukemia. This idea is supported by genome wide data that identify additional recurring genetic alteration in TEL-AML1 ALL such as deletions in the B cell master regulators PAX-5 and EBF, as well as deletion of the second copy of TEL (Mullighan et al., 2007; Parker et al., 2008). Another frequent translocation present in 70% of high-risk infant leukemia and with poor prognosis is the translocation t(4,11) resulting in the chimeric protein MLL (mixed- lineage leukemia) and AF4 (fused gene from chromosome 4). The MLL gene encodes a methyltransferase protein which methylates lysine 4 on histone H3 (H3K4) to regulate patterns of gene expression, particularly those of the homeotic gene family HOX, that are involved in development (Krivtsov and Armstrong, 2007). Despite recent advances MLL-AF4-driven leukemogenesis remains difficult to model in mice to accurately recapitulate the disease phenotype (Stam, 2012). More than 40 translocations involving the MLL1 and AF4, AF9, ENL, AF10 and AF6 genes have been found (Horton and Williams, 2006; Meyers et al., 1996). Many of the

MLL fusion proteins are located in protein complexes that regulate transcriptional elongation, which may be in part responsible for transcriptional deregulation and leukemogenesis. Moreover MLL rearranged leukemia highlight the potential importance of epigenetic alterations in the pathogenesis of ALL as well as potential therapeutic role of epigenetic modifying drugs (Mullighan, 2012).

The translocations involving the E2A gene in B-ALL are two: one results in the E2A- PBX1 (pre B cell leukemia homeobox1) fusion protein and the other one E2A and hepatic leukemia factor (HLF) (Seidel and Look, 2001). E2A-PBX-1 is found both in childhood leukemia (5-6% of ALLs and 23% of pediatric leukemia) and in adult leukemia (1-3%) where it has a much poorer outcome (Aspland et al., 2001). The transcription factor E2A is encoded by the Tcfe3 gene and it is needed in the B lineage commitment (Zhuang et al., 1994). PBX1 is a homeobox-containig protein that is required for the development of lymphoid precursors (Sanyal et al., 2007). The chimeric transcription factor E2A-PBX1 has two simultaneous effects: it disrupts one allele of both E2A and PBX1 and leads to the mis-expression of PBX1 under the control of E2A regulatory sequences. In particular E2A-PBX-1 chimeric protein may function to alter the expression patterns of PBX target genes, such as HOX genes, as well as sequestering E2A co-activators, leading to the repression of E2A target genes and to uncontrolled cell cycle progression (Aspland et al., 2001). The fusion protein E2A-HLF is present at very low frequency in both children and adult (0.5- 1%), and its prognosis is highly unfavorable (Campos-Sanchez et al., 2011). HLF is a member of the PAR-bZIP (proline and acid amino acid rich basic leucine zipper) transcription factor family expressed in liver,

kidney, lung and nervous system but is not expressed in the lymphoid compartment (Hunger et al., 1992). Even though it is clear that the chimeric protein E2A-HLF affects lymphocyte development rendering B cells susceptible to malignant transformation, the molecular mechanisms are not totally clear yet (Seidel and Look, 2001). It seems to aberrantly regulate LMO2 and BCL2 genes that control cell death in lymphoid progenitors (de Boer et al., 2011).

The BCR-ABL1 fusion protein is much more frequent in adults (25%) than in children (3-5%) (Mancini et al., 2005). The BCR-ABL1 fusion gene is formed by a translocation between chromosomes 9 and 22, which also results in an abnormally short chromosome 22 (the Philadelphia chromosome; Ph) (Ribeiro et al., 1987). ABL is a tyrosine kinase implicated in signal transduction important for many biological processes such as cell differentiation, cell division, cell adhesion and stress response. The fusion protein resulting from the BCR-ABL translocation leads to the activation of multiple signaling pathways contributing to leukemogenesis (Tybulewicz et al., 1991). Moreover, the transcription factor IKAROS, required for the development of all lymphoid lineages, is commonly found to be deleted in BCR-ABL1 lymphoid leukemia (Mullighan et al., 2008).

Successful treatment of leukemia typically spans 2-2.5 years and comprises three phases: induction of remission, consolidation and maintenance. Current treatments typically include the use of chemotherapeutic drugs such as glucocorticoid (prednisone, or dexamethasone), vincristine and asparaginase with or without anthracycline in different combinations and dosage. Patients with BCL-ABL1 translocation are treated with tyrosine kinase

inhibitors such as imatinib and dasatinib. In addition, allogeneic hematopoietic stem cell transplantation is an option for very high-risk patients. However, most of the drugs used to treat leukemia were developed before the 1970s. Moreover emerging evidence show that ALL often relapses by the outgrowth of sub-clones harboring novel genetic alterations that confer chemoresistance. Thus, efforts are necessary to improve diagnosis and development of targeted therapy (Inaba et al., 2013)

7.2 LYMPHOMA

Lymphomas are primary divided into two groups:

- Hodgkin's lymphoma (HL) characterized by the presence of multinucleated Reed–Sternberg cells (RS cells)
- Non-Hodgkin's lymphoma (NHL)

About 95% of NHL are of B-cell origin and are classified into 15 different types according to the Word health organization (WHO) (Vardiman et al., 2009). This classification is relevant not only in terms of lymphoma pathogenesis but also from the clinical point of view because different types of lymphoma have very different clinical behaviors and therefore may require diverse treatment strategies (Kuppers, 2005). Recent studies on normal and malignant B expression profiles have provided new insights into the phenotype of GC B cells and the transcriptional programs that establish the distinct phenotypes, helping in clarify the cellular origin of B cell lymphomas. B-NHLs are classified according to the differentiation stage of the malignant cells. The rationale for such a classification is based on the observation that malignant B cells seem to be frozen at a particular

differentiations stage, which appears to reflect their origin. One emerging concept in the field is that, with the exception of relatively rare subtypes of lymphomas, most type of B cell lymphomas are derived from GC or post GC B cells (Klein and Dalla-Favera, 2008; Kuppers, 2005). A hallmark of many types of lymphoma is the reciprocal chromosomal translocations involving one of the immunoglobulin loci and a proto-oncogene. As a consequence, the oncogene comes under the control of an active immunoglobulin locus, causing deregulated, constitutive expression of the translocated gene in B cells. Translocations found in B-NHL differ from the ones found in most acute leukemias containing fusion proteins (Kuppers, 2005).The process of somatic hypermutation (SHM) and class switch recombination (CSR) happening during the germinal center reaction contributes to lymphoma pathogenesis not only by causing chromosomal translocations, but probably also by targeting non IgG genes. This could be the reason why most B cell lymphomas derive from the GC and could also partially explain why B cells are more prone to undergo malignant transformation than T cells that do not undergo such processes (Montesinos-Rongen et al., 2005). Other transforming events have been implicated in B cell malignancies such as mutations in tumor-suppressor genes (TP53 and ikBa), genomic amplifications such as of REL and translocations not involving Ig loci (AP1-Malt1). Finally, viruses may also be involved in the transformation of B cells. The most well known example is Epstein-Barr virus (EBV), which is found in nearly all endemic Burkitt's lymphomas (Kuppers, 2005).

A common denominator of chromosomal translocations associated with B-NHL is the transcriptional dysregulation of

genes that control GC B cell development, or the ectopic expression of genes not normally expressed in a particular developmental stage of mature B cells (Fig. 7). One example is the t(14;18) chromosomal translocation involving the igH and the BCL-2 genes characteristic of follicular lymphoma (Jager et al., 2000). By histology these lymphomas have a follicular architecture and molecularly show ongoing IgV SHM. As a result of the translocation the proto-oncogene BCL-2 is under the control of the active IgH locus and becomes overexpressed at the centroblast stage, where it is normally silent, promoting cell proliferation by decreasing the propensity to undergo apoptosis (Klein and Dalla-Favera, 2008). In contrast, the BCL-6 gene is highly expressed in and required for GC formation but its expression is turned off in post GC differentiation to allow the maturation of B plasma cells. Deregulation of BCL-6 may result in a block in post-GC differentiation resulting in a pro-proliferative phenotype. Chromosomal translocations involving the BCL-6 gene are commonly associated with germinal center diffuse large B cell lymphoma (GC-DLBCL) (Ye et al., 1993).

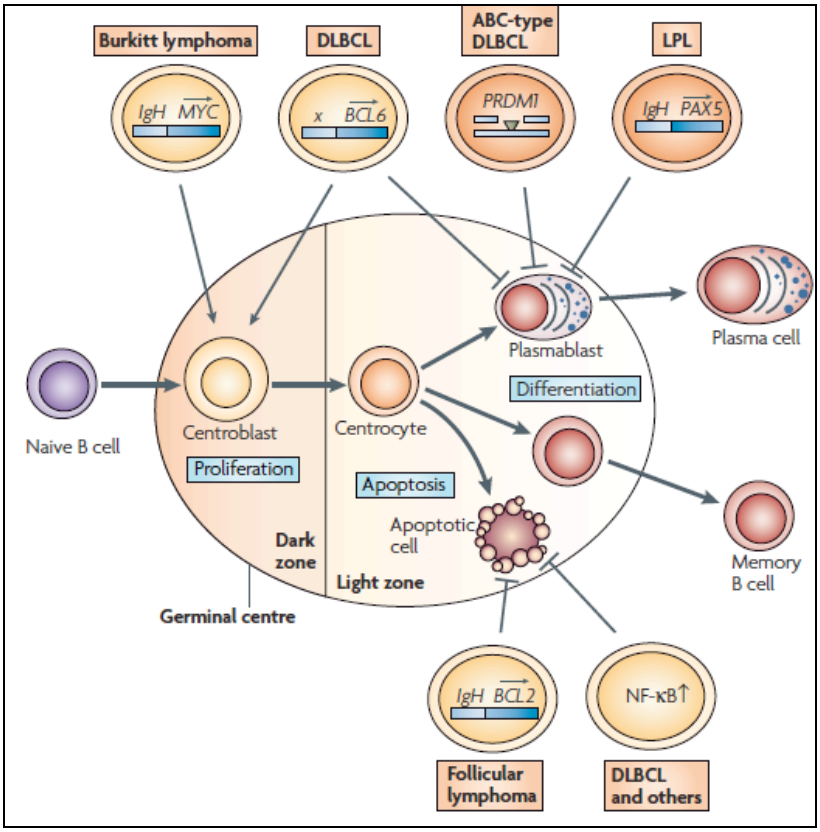

Fig.7 Stages during human B cell differentiation at which genetic aberrations generate lymphoma cells (adapted from Klein and Dalla-Favera, 2008).

A block in post-GC differentiation is also observed in chromosomal translocations affecting PAX-5 found in lymphoplasmacytoid lymphomas (LPL). Pax-5 expression is required for the commitment of lymphoid progenitors to the B cell lineage and its expression through B cell development helps maintaining B cell identity. However, Pax-5 is not expressed in plasma cells. The translocation of Pax-5 and the IgH locus prevents Pax-5 silencing and therefore blocks the terminal differentiation of B cells into plasma cells (Iida et al., 1996). Translocation of MYC into the immunoglobulin heavy or light

chain loci are associated with 100% of Burkitt lymphomas (BL) and up to 10% of DLBCL (Alizadeh et al., 2000 {Klein, 1995 #2030). Gene expression profiling of GC B cells revealed that they do not express MYC. The ectopic expression of MYC in GC B cells contributes to lymphomagenesis both directly by controlling cell growth and indirectly by favouring the accumulation of additional genetic alterations (Vafa et al., 2002);Dominguez-Sola, 2007 #807}. The possible role of additional genetic alterations that may contribute to lymphomagenesis found in other types of cancer, such as deletions, mutations and amplifications remain to be characterized for B-NHL. One indication that such alterations can contribute to the formation of B cell tumors is the alteration of the gene PR domain containing 1 (PRDM1). PRDM-1 is expressed in a subset of germinal center B cells and in all plasma cells, and is required for terminal B cell differentiation. PRDM-1 alterations such as gene truncations, nonsense mutations, frame shift deletions, and splice site mutations that generate aberrant transcripts encoding truncated proteins have been found in a subset of DLBCL called activated B-cell type (ABC-DLBCL). These findings point toward a tumor suppressor role of PRDM1, whose inactivation may contribute to lymphomagenesis by blocking post-GC differentiation of B cells into plasma cells (Tam et al., 2006) (Pasqualucci et al., 2006).

Finally, also activation of signaling pathways, such as involving NF-kB in centroblast cells, where it is normally silenced, has been implicated in DLBCL pathogenesis. However no specific genetic alterations have been identified that can explain the deregulated activation of this pathway (Basso et al., 2004; Shaffer et al., 2001).

Currently, chemotherapy, radiation and anti-CD20 antibody treatment are the mainstays of B-cell lymphoma treatment. However, the fact that a substantial number of patients are eventually not cured justifies the search for novel and more effective therapeutic approaches (Shaffer et al., 2012). Promising new strategies involve the recently described combination of CD47 and CD20 antibodies can abrogate the tumorigenicity of human B-cell lymphoma in immunodeficient mice (Chao et al., 2010). In addition, small molecules that block BCL6 co-repressors have been developed and are effective in experimental models of B-cell lymphoma (Cerchietti et al., 2010). Other novel therapies include the exogenous administration of soluble tumor suppressors (Oricchio et al., 2011).

8. TRANSDIFFERENTIATION OF MURINE B CELLS INTO MACROPHAGES

Previously, our group reported that forced expression of C/EBPα in primary mouse B cells can convert them into macrophage-like cells in a fast (4-5 days) and highly efficient way *in vitro* (65% of pre-B and 35% of mature B cells). Transdifferentiation has been shown to occur also *in vivo*. Thus, B cell precursors (B220+) from lineage tracing mice (CD19Rosa26 EYFP), in which B cells are permanently labeled in green, were purified and infected with C/EBPα hCD4 retrovirus and injected into sublethally irradiated Rag2-/- γc -/- mice. 6 days after the injection 51% of the C/EBPα EYFP+ cells were CD19-Mac-1+ in the bone marrow and 32% in the spleen. At the molecular level, C/EBPα inhibits the B cell program by inhibiting the B cells factor Pax-5, leading to the

downregulation of its target CD19. Simultaneously, by interacting with the ETS family transcription factor PU.1, it upregulates myeloid markers such as Mac-1. These two processes can be uncoupled since in PU.1 deficient pre-B cells C/EBPα induce CD19 downregulation but not Mac-1 activation (Xie et al., 2004). However, the primary B cell system is heterogeneous, the cell number is limited and the culture require stroma cells, thus the study of the molecular events underlying the transdifferentiation process is difficult to study. Therefore, a robust transdifferentiation system was developed, consisting of a clone of pre-B cell line (HAFTL) expressing an inducible form of C/EBPα fused to the estrogen receptor (ER). The C/EBPαER expressing cells can be converted by b-estradiol (E2), in the absence of stroma, into macrophages like cells at 100% efficiency in 2 to 3 days. The obtained macrophages cells are large, highly migratory, phagocytic and exhibit inflammatory responsiveness. After induction of C/EBPα macrophages genes become upregulated and B cells specific transcription factors and genes become downregulated. Among the downregulated genes, cell cycle genes and chromatin remodeling factors such as the polycomb complex II component Ezh2 and the DNA methyltransferase Dnmt3b have been found. Moreover, these cells become transgene independent within 1 to 2 days (Bussmann et al., 2009).

The use of this robust and efficient transdifferentiation system permitted to study the question if during transdifferntiation the cells pass trough and intermediate progenitor stage of even retrodifferentiate to a pluripotent state before becoming macrophages. Studies performed in our lab have shown that

during the transdifferentiation process there is a transient reactivation of immature myeloid markers, as well as low levels of progenitor markers (Flt3 and Kit) at the mRNA level. Importantly, however, we were not able to detect re-expression of cell surface markers that characterize HSCs and progenitors (HSCP), even when C/EBPα was activated in pre-B cells under culture conditions permissive for HSCP cell growth or when C/EBPα was activated in a time limited fashion. Taken together these data have shown that the B to macrophage switch is a direct process that does not involve retrodifferentiation (Di Tullio et al., 2011). Further studies performed by our group have shown that the cell cycle is not strictly required during immune cell transdifferentiation. In particular, time-lapse experiments have shown that after induction of C/EBPα approximately 90% of the cells divide one or twice, while 8% do not at all before acquiring a macrophage phenotype. Importantly, the non dividing subset express the highest level of C/EBPα and is the fastest in differentiating, suggesting that high level of C/EBPα accelerate both the switching process as well as cell cycle arrest. (Di Tullio and Graf, 2012). More mechanistic insights came from the study of the role of chromatin modifications, such as DNA methylation during the transdifferentiation process. Unexpectedly, cell lineage conversion occurred without significant changes in DNA methylation in key B cell and macrophage- specific genes, showing that transdifferentiation is different from reprogramming into pluripotent cells in which DNA demethylation plays an essential role in the reactivation of pluripotent genes. However, active (H3K9ac, K14ac and H3K4me3) and repressive (H3K27me3) histone modification marks changed according to

the expression levels of these genes (Rodriguez-Ubreva et al., 2012). More detailed studies using the inducible pre-B cell line have shown a role for the enzyme Tet2, which hydroxylates methylated cytosine residues. C/EBPα activates Tet2 expression, resulting in rapid hydroxymethylation of the promoters of approximately 60 myeloid target genes which as a consequence become more rapidly de-repressed during transdifferentiation (Kallin et al., 2012).

More recent work performed on B to macrophage transdifferentiation system has demonstrated a more general role for histone deacetylases 7 (HDAC7) during myeloid to lymphoid branching point during differentiation. HDAC7 is expressed in pre-B cells and functions as a transcriptional co-repressor together with MEF2c transcription factors to silence myeloid genes. During the conversion from pre B cells to macrophages HDAC7 expression is downregulated. Importantly exogenous expression of HDAC7 in pre-B cells results in a block of Mac-1 expression and interferes with functional characteristics of transdifferentiated macrophages (Barneda-Zahonero et al., 2013).

Aims

The observation that transdifferentiated macrophages are phagocytic and become quiescent, acquiring a normal phenotype (Bussmann et al., 2009), and that they do not give rise to tumors when injected into mice (Bussmann, L. unpublished data) raised the possibility to develop a differentiation strategy for the therapy of human B cell leukemias and lymphomas

- Test whether human B leukemia/lymphoma cell lines can be transdifferentiated into macrophages by C/EBPα, and if they lose tumorigenicity.

- Screening human lymphoma cells for small molecules able to convert B cells into macrophages.

PART II

RESULTS

Chapter 1

C/EBPα induces highly efficient macrophage transdifferentiation of B lymphoma and leukemia cell lines and impairs their tumorigenicity

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Chapter 2

Screening of human lymphoma cells for small molecules able to convert B cells into macrophages

We previously showed that is possible to transdifferentiate human leukemia and lymphoma cell lines into quiescent macrophages *in vitro*. Moreover we demonstrated that the activation of C/EBPα *in vivo* in a Burkitt lymphoma clonal cell line and in a less mature acute lymphoblastic leukemia (B-ALL) cell line, inhibits/delays tumorigenicity. The results obtained overexpessing C/EBPα in human leukemia and lymphoma cell lines encouraged us to screen for small molecules that could induce transdifferentiation of human tumorigenic B cells into non-dividing macrophages. Such compounds could be tested for their ability to block leukemia *in vivo*, providing a novel therapeutic approach to treat B cell malignancies.

Generation of a myeloid gene reporter system

To have a fast and clear read-out for the screening, we generated a reported system based on the lysozyme promoter dsRed reporter (on/off) (Fig.1). Lysozyme is most highly expressed in granulocytes and monocyte/macrophages and undetectable in B cells (Clarke et al., 1996). Moreover, the lysozyme gene has been shown to be a direct target of C/EBP transcription factors (Ness et al., 1993). We therefore cloned the human lysozyme promoter fused to the dsRed reporter in a modified PHAGE lentivirus. The construct was then used to produce virus and to infect the BLaER1 cell line (containing the C/EBPα ER GFP). To establish a clonal cell line expressing high levels of dsRed, GFP^+ cells were sorted and seeded in 96 well plates in which they were expanded for a few days. Each clone was split into two wells and tested for the appearance of dsRed

after induction with E2. One clone (#1) was chosen for further validations.

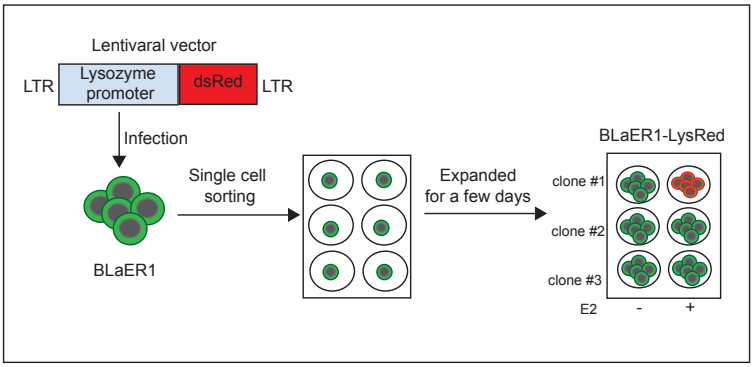

Fig.1 Strategy used to generate the myeloid gene reporter system.

Validation of the myeloid reporter system

To test if the BLaER1-LysRed system could be used for the screening of compounds able to induce the transdifferentiation of malignant B cells into macrophages we induced the cells for various times with E2 and tested the cells for dsRed fluorescence by fluorescent microscopy and FACS (Fig.2). Images were collected at two different days after induction with E2 (d2 and d4) and compared to uninduced cells (d0) (Fig.2A). FACS analyses were performed at the same time points using the BLaER1 GFP expressing cells as a control (Fig.2B). In both cases we could detect the appearance of dsRed and thus the activation of the lysozyme promoter as soon as 2 days after induction. Further analysis comparing the expression of Mac-1 and dsRed during transdifferentiation revealed that the expression of dsRed increased over time, reaching a maximum expression 4 days after induction (Fig.2C). Moreover, the

appearance of dsRed precedes the expression of Mac-1 antigen. Therefore the generated BLaER1-LysRed cell line is a suitable system to screen molecules able to convert B cells into macrophages. To select libraries of compounds that could activate the macrophage program in malignant B cells we chose the following 5 pathways involved in macrophage differentiation and/or function: the Toll-like receptor pathway, Mitogenic Ets Transcriptional Suppressor pathways (METS), NF-kB signaling pathway, TNF/stress related signaling and IFN gamma signaling pathway. Using the BioCarta pathways program we identified 44 possible targets belonging to the 5 different pathways mentioned above. Using a ligand-based *in silico* screening (Chemotarget S.L.) a total of 409 available compounds were selected. Among the focused libraries we also included 80 compounds targeting tyrosine kinase inhibitors (Tocriscreen Kinase inhibitors) as well as 84 putative AuroraA/B inhibitors (Chemotarget S.L.). We also included in our library 1200 pharmacologically active small molecules and 320 phytochemical compounds from Prestwick libraries (Prestwick Chemical). Moreover, we also included 25 compounds selected from the literature described to be effective in enhancing iPS cell reprogramming.

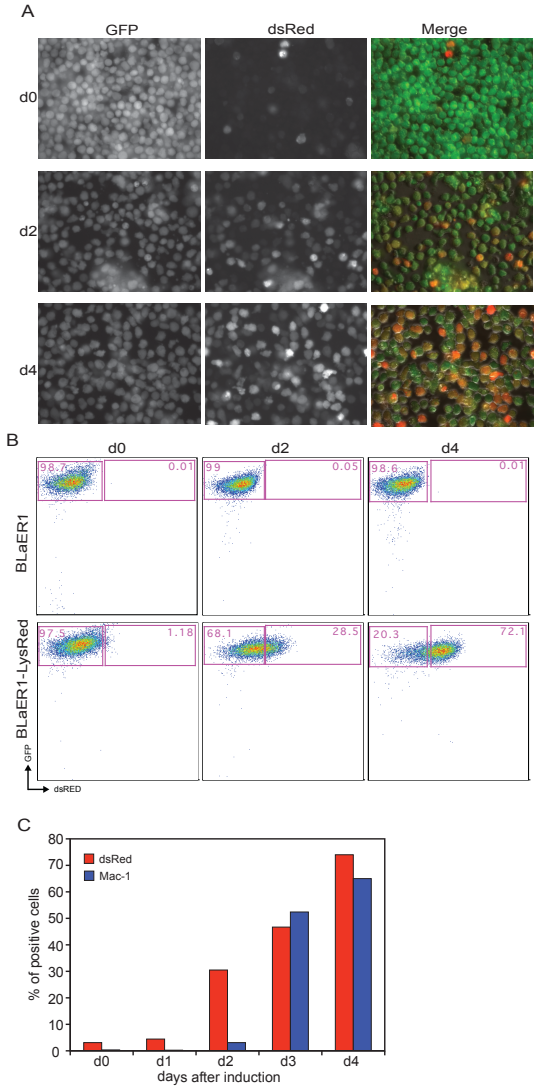

Fig.2 Validation of the myeloid gene reporter system. (A) Pictures of uninduced, 2 and 4 days induced cells were acquired in green and red fluorescence channel and the merge was performed using Adobe Photoshop. (B) FACS plots of BLaER1 and BLaER1-LysRed cells induced for 2 and 4 days, compared to uninduced cells, indicating the percentage of GFP/dsRed+ cells. (C) Graph showing the kinetics of Mac-1 and dsRed expression in induced BLaER1-LysRed cells.

Screening

A total of 2093 compounds were tested at two concentrations (1 and 10 μM) and images were acquired at two time points (d2 and d5). All the experiments were performed in duplicate. From the screening, based on strictly standardized mean difference (SSMD) analysis, 30 primary hits were identified. Among these compounds, 21 were estradiol- or tamoxifen-like analogues. To validate the remaining 9 primary hits, compounds were tested in a second screen and only 3 passed the validation test. The 3 validated compounds are natural products belonging to the phytochemicals Prestwick library. The structure of the confirmed compounds, Vochysine, Genistin and Coronaridine does not present any evident similarity with 17-β-estradiol of tamoxifen (Fig.3). However, a more detailed analysis of available literature revealed that one of the compound, Genistin, competes with estradiol for binding to the estrogen receptor (ER) (Wang et al., 1996).

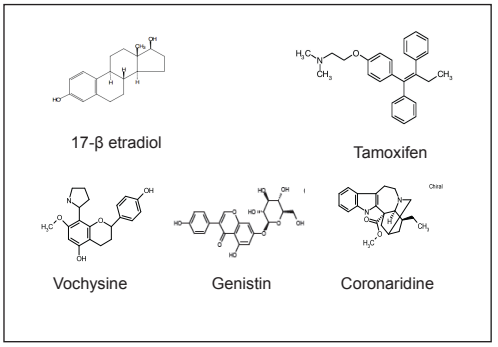

Fig.3 Chemical structure of Vochysine, Genistin and Coronaridine, as well as 17-β estradiol and tamoxifen.

The half maximal effective concentration (EC_{50}) of the 3 compounds was measured as shown in Fig.4A.

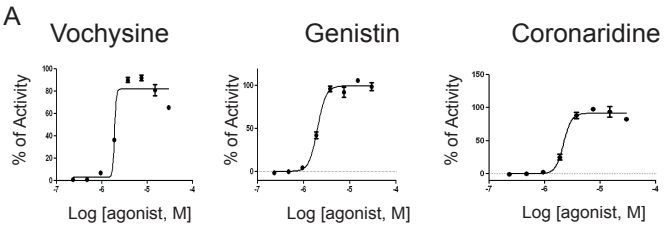

Fig. 4 The half maximal effective concentration (EC_{50}) curves. (A) EC_{50} for each compound was calculated using 8 serial dilutions.

To understand if the effects of the selected compounds were due to specific activation of the myeloid program, rather then simply activating the reporter transgene, we tested the compounds on the parental lymphoma cell line, Seraphina, by FACS. We also included in our validation experiments a less mature B cell line, RCH-ACV, previously shown by us to be susceptible to the overexpression of C/EBPα. We included the compound Genistin as a positive control and tested the 3 compounds on the BLaER1-LysRed cell line, as a positive control. Cells were incubated with 10 μM of the compounds and analyzed 5 days after by monitoring changes in expression of CD19 and Mac-1 antigen as well as changes in cell size (FSC) and granularity (SSC). For the BlaER1-LysRed cell line, we first checked the activation of the lysozyme promoter, by quantifying the dsRed fluorescence by FACS. As show in figure 5, all the compounds tested were able to induce the expression of the lysozyme in the majority (>90%) of the cells after 5 days.

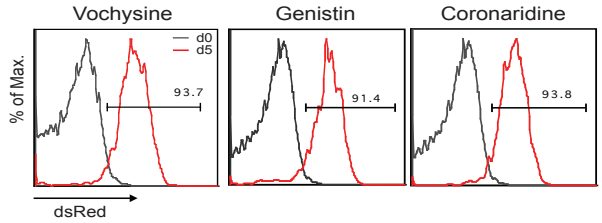

Fig.5 Quantification of dsRed fluorescence of BLaER1-LysRed cells after incubation with the three effective drugs. (A) Histograms represent the red fluorescence of uninduced (black line) and BLaER1-LysRed cells treated for 5 days with the compounds (red line).

FACS analysis of Seraphina and RCH-ACV cell line incubated with the compounds for 5 days did not show any downregulation of CD19. A slight increase in Mac-1 expression was observed in RCH-ACV cells treated with Genistin. No upregulation of Mac-1 could be detected in Seraphina cells treated with the 3 compounds. However, the BlaER1-LysRed cell line showed slight upregulation of Mac-1, but not downregulation of CD19 (Fig.6A). To further confirm any possible effects of the drugs we also checked the cell size (FSC) and granularity (SSC) of the treated cells. The BlaER1-LysRed cell line showed an increased cell granularity (SSC), but not cell size (FSC) after incubation with the compounds. We could also detect a slight increase of granularity in the two tumorigenic cell lines (Seraphina and RCH-ACV) treated with Coronaridine. Morphological analysis of the cell lines incubated with the compounds did not show any change in terms of adherence, a characteristic of macrophages (data not shown).

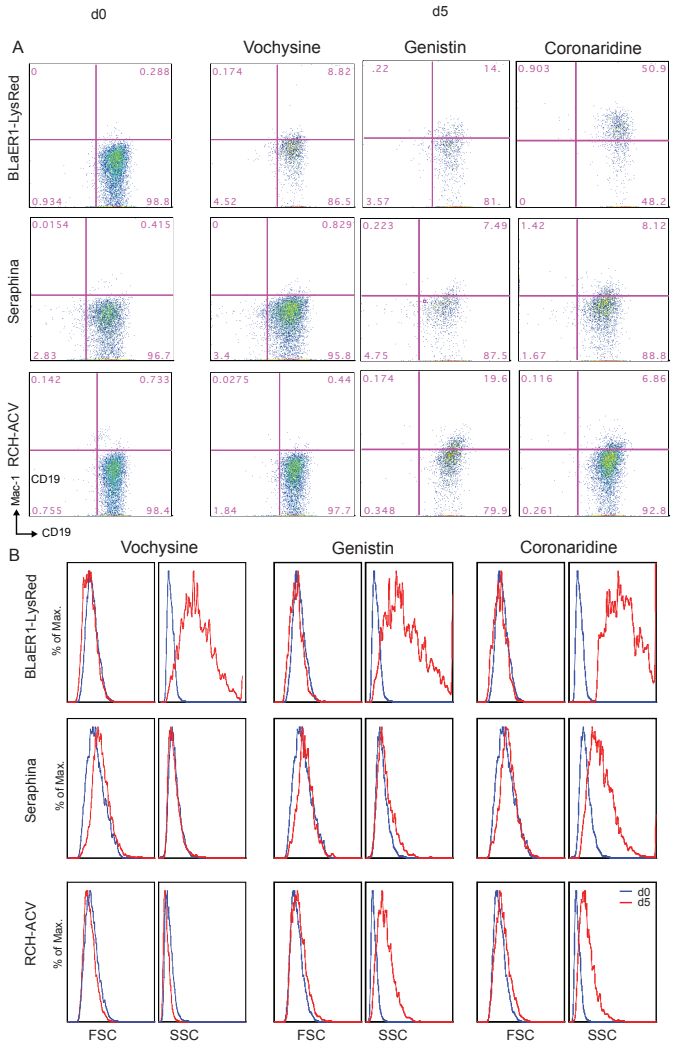

Fig.6 Validation of the compounds by flow cytometry monitoring Mac-1/CD19 expression as well as cell size and granularity. FACS plots represents BlaER1-LysRed, Seraphina and RCH-ACV cells incubated with Vochystine, Genistin and Coronaridine for 5 days. (A) Plots represent CD19 and Mac-1 expression of treated (d5) compared to untreated (d0) cell. (B) Histograms represent cell size (FSC) and granularity (SSC) of untreated (d0, blue line) and treated cells (d5, red line).

To further test the possibility that the effects seen in the BlaER1-LysRed cell line was not due to the activation of the exogenous

ER we incubated mouse C11 cells, containing the C/EBPαERhCD4, and the pre-B cells line HAFTL with the 3 compounds at 10 μM for 5 days. FACS analysis of Mac-1 and CD19 as well as FSC and SSC revealed that only C11 cells are affected by the treatment and upregulate the macrophage marker Mac-1, whereas the parental cell line HAFTL do not show any detectable change in antigens expression as well as cell size and granularity (Fig.7). Taken together these data suggests, that none of the three candidate compounds activate the endogenous macrophage program and that Vochysine and Coronaridine may activate the estrogen receptor, although this has not been reported.

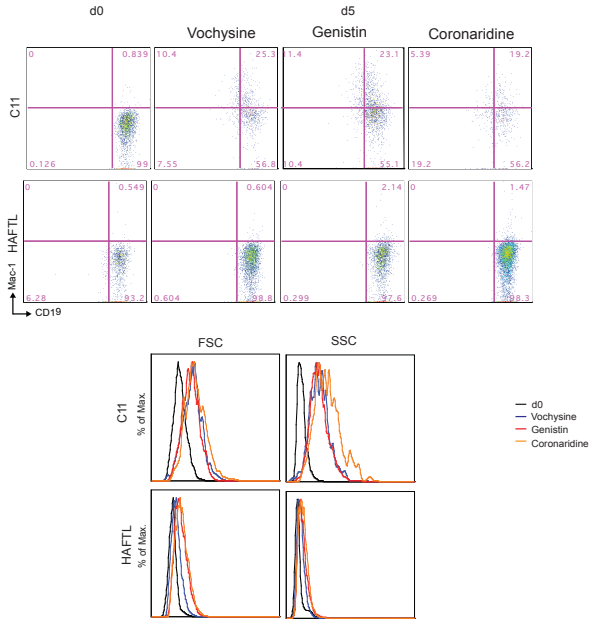

Fig.7 Validation of the compounds on mouse cell lines. (A) FACS plots representing the changes in Mac-1 and CD19 expression of C11 and HAFTL cell lines incubated with the drugs. (B) Histograms represent changes in FSC and SCC of C11 and HAFTL cells untreated (black line), and treated with Vochysine (blue line), or Genistin (red line) or Coronaridine (orange line).

MATERIAL AND METHODS

Cloning

The human lysozyme promoter -3500 to +25 was amplified from HeLa genomic DNA with the following primers: (FW) CTCAACCCCGAGTAGCTG and (REV) AGGACTGCTTGAGACCAGGA. The 3.5 kb fragment was cloned in fusion with the dsRED into a modified lentivurs PHAGE vector.

Cell culture and infection

The culture of human leukemia and lymphoma cell lines (Seraphina and RCH-ACV) and derivatives (BLaER1 and BLaER1-LysRed), virus production and infection was performed as previously described (Rapino et al., 2013). The mouse pre-B cell lines HAFTL and C11 were cultured as previously described (Bussmann et al., 2009)

Flow cytometry

FACS analysis were performed at the time points indicated using directly conjugated antibodies against Mac-1 (APC) and CD19 (APC-Cy7) (BD Pharmingen) for human cell lines and using antibodies against mouse Mac-1 (PE-Cy7) and CD19 (APC) (BD Pharmingen). DAPI at 1 µg/ml was used as viability markers. Samples were analyzed on the LSRII flow cytometer (BD Biosciences, San Diego, CA) and data analyzed with FlowJo software (Tree Star, Ashland, OR)

Compounds library

Chemical (PW001) and phytochemical (PW002) libraries were purchased from Prestwick Chemical. The chemotarget (EN001) *in silico* selected compounds and kinase inhibitors (TO001) library were purchased from Enamine and Tocriscreen respectively. Selected compounds (DV001) were purchased from TocrisBioscience. The Aurora A and B inhibitory compounds (AUR001) were *in silico* selected based on the aurora kinase pharmacophore model and purchased from different providers . All the compounds, when not bought already re-suspended, were diluted at a concentration of 10 mM in DMSO.

Screening

Assay 96 wells optical black plates (ref.165305 NUNC) were labeled with a barcode identifier and coated with 50 μl of poly-D lysine (100ug/ml) and incubate at 37 C for 30 minutes. After removal of the coating, wells were washed with sterile H_2O using the Platewasher ELx405 (BioTek) and dried overnight at room temperature. BlaER1-LysRed cells were seeded using the Multidrop 384 microplate dispenser (Thermo scientific) at concentration of 20.000 cells/wells in 100 μl of complete medium (RPMI 1640, 10% FBS and P/S and l-Glu) in the presence of 10ng/ml of hr M-CSF and hr IL-3 (Peprotech). Compounds were added to the plates using the E4XLS Eletronic pipette at the final concentration of 1 and 10 μM. Each 96 well plate contain positive control (medium plus E2 and cytokines) or negative control (medium alone). Each plate was tested in duplicates.

Image acquisition and data analysis

Images were acquired 2 and 5 days after addition of the compound using the microscope MetaXpress (from Molecular Devices). 5 different sites were acquired per each well and the mean value was calculated excluding the maximum and the minimum value from the dataset. Strictly standardized mean fluorescence (SSMD) was calculated according to the formula below. Hit with an SSMD >3 in the two replicates were considered positive.

$$\hat{\beta} = \frac{\log_2 (x_i - median(x_n))}{\sqrt{2}\ MAD(\log_2 x_n)}$$

x_i - current sample value

x_n – negative controls

Concentration-response curve

30 mM solutions were serially diluted 1:2 in DMSO to generate a 8-points concentration response curve for the 3 compounds tested: Vochysine, Genistin and Coronaridine. The percentage of activity was calculated according to the formula:
% activity = (well raw counts – avg neg ctrl raw counts)/(avg pos ctrl raw counts – avg neg ctrl raw counts)*100.
EC_{50} values were calculated using Prism using Dose-response: Stimulation

- Log(agonist) vs. response -- Variable slope (four parameters)

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PART III

DISCUSSION AND CONCLUSIONS

Discussion

The induction of differentiation as a therapeutic approach for cancer is a concept postulated more than three decades ago. This was largely based on the findings that erythroleukemic cell lines can be induced to terminally differentiate with compounds such as DMSO and HMBA, and myeloid leukemia cell lines differentiate after treatment with retinoids and vitamin D (Nowak et al., 2009). Subsequently it was shown that administration of all-trans retinoic acid to patients with acute promyelocytic leukemia (APL) induces granulocytic differentiation and is very effective therapeutically (Waxman, 2000). The knowledge about transcription factors governing hematopoietic differentiation and the discovery of mutations or mis-regulation in leukemia, led to the strategy to use master regulator transcription factors to overcome the block in differentiation characteristic of malignant cells. Due to its role in myeloid lineage differentiation, C/EBPa has been used to convert CD34+ bone marrow derived precursors from AML into more mature, quiescent myeloid cells (Schepers et al., 2007). Studies performed with the myeloid cell line HL60 have shown that the compound CDDO is able to increase the translation of the full-length p42 isoform of the C/EBPα, thus inducing partial differentiation into granulocytes (Koschmieder et al., 2007). Differentiation induction has not been reported for lymphoid neoplasms and currently treatments for B-cell lymphoma and leukemia consist in chemotherapy, radiation and anti-CD20 antibody. However, the fact that a substantial number of patients are eventually not cured justifies the search for novel and more effective therapeutic approaches (Shaffer et al., 2012).

Due to the powerful role of C/EBPα in the differentiation of malignant myeloid cells (Schepers et al., 2007) and its capacity

to induce the transdifferentiation of murine B cells into quiescent macrophages at high efficiency (Bussmann et al., 2009; Xie et al., 2004), in the current work we investigated whether human lymphoid neoplasm-derived cell lines are susceptible to C/EBPα-induced transdifferentiation into macrophages and if so, what is the impact on their tumorigenicity.

Our data have shown that the ectopic expression of C/EBPα can induce highly efficient conversion of selected human lymphoma and leukemia B-cell lines into macrophages. The reprogrammed cells are functional and in addition, the Burkitt lymphoma cells retained their macrophage phenotype even after the transgene was inactivated, showing that the process observed corresponds to bona fide transdifferentiation. Importantly, the tumorigenicity of transdifferentiated lymphoma and leukemia cell lines was impaired after transplantation into immunodeficient mice, even when C/EBPα was activated *in vivo* (Fig.1).

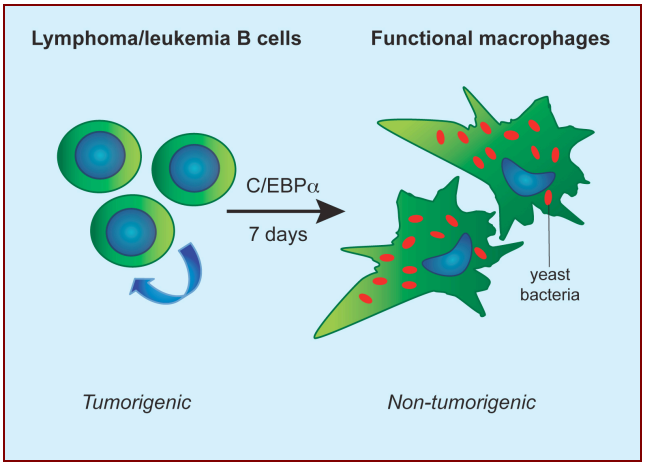

Fig.1. The conversion of human lymphoma and leukemia B cells into functional and quiescent macrophages inhibits their tumorigenicity

We tested 23 cell lines derived from patients with various B-cell lymphoma and leukemia subtypes, for their susceptibility of conversion into macrophages and found that 19 responded at least partially while 4 did not respond at all. Closer analysis revealed, that with one exception, no C/EBPA protein was detectable in the 4 non-responder lines, explaining their 'resistance'. The major problem in transducing human B cells, is the low efficiency of infection. We used two different inducible vectors to overexpress C/EBPα: a retrovirus and a lentivirus. One disadvantage in using retrovirus vectors is their inability to infect non-dividing cells. In fact even in slowly dividing cells, this system only gave low infection rates. We also tried a lentiviral system that overall gave a better infection efficiency even though for some cell lines in particular we were not able to efficiently overexpress C/EBPα.

Moreover, analysis of the partially responding cells suggested that responsiveness is directly correlated to the transgene levels, a phenomenon described also for iPS cell reprogramming (Polo et al., 2012). In fact, cells with the highest levels of C/EBPα exhibited the highest levels of Mac-1 antigen. Furthermore, in many partial responders or non-responders we observed a gradual decrease over time of GFP or tdTomato used as indicators of C/EBPα expression during transdifferentiation that points towards silencing or instability of the transgene. In the light of the low infection efficiency of B cells and the importance of high doses of C/EBPα for transdifferentiation it is not surprising that the majority of the cell lines tested only partially responded to the overexpression of C/EBPα. Interestingly, the silencing of CD19 appears to require lower C/EBPα levels than

Mac-1 upregulation, in fact the downregulation of the B cell antigen was the main effect that we observed in the partial responder lines. Our evidence for the conclusion that partial responders undergo a transient transdifferentiation came from qRT-PCR experiments. Using this technique we were able to show that 5 key macrophages and 5 B cell genes were up- and downregulated respectively, after overexpression of C/EBPα in partially responding cell lines, although the levels of the transgene were too low to induce the expression of these markers at the protein level. Moreover, apart from the dosage dependent effects of C/EBPα, it is known that cell lines kept in continuous culture for prolonged periods of time accumulate DNA methylation and chromosome rearrangements (Mestre-Escorihuela et al., 2007). This could also explain why one of the cell lines analyzed having detectable levels of C/EBPA did not respond with cell conversion. Taken together, we conclude that the majority of lymphoma and leukemia cell lines tested, which belong to a broad spectrum of neoplasms, respond at least partially and transiently to the lineage reprogramming effects of C/EBPα, and that cells with a sustained and high level transgene expression undergo an efficient macrophage transdifferentiation.
Our results are in agreement with work of others that showed that transdifferentiation and reprogramming in human cells takes longer to achieve compared to mouse cells (Ladewig et al., 2013), in fact induced transdifferentiation of human leukemia and lymphoma cell lines was slower by about 3-4 days compared to mouse pre-B cell lines expressing the same inducible construct (Bussmann et al., 2009). Moreover, considering the fact that human cells have been found to be generally much more

resistant to transcription factor induced reprogramming than mouse cells (Stadtfeld and Hochedlinger, 2010), the observed high conversion frequency of the Seraphina Burkitt lymphoma and the RCH-ACV B-ALL line described is remarkable. This high efficiency could be due to the close differentiation relationship between the myeloid and the B lymphoid lineage and to the powerful role of C/EBPα in the specification of the myeloid lineage. How C/EBPα acts to induce transdifferentiation and which chromatin modifying enzymes it needs to promote chromatin remodeling is currently under investigation in our lab.

To determine whether transdifferentiated cells are still tumorigenic we performed several *in vivo* experiments injecting uninduced and induced cells into immunodeficient Rag2 -/-γc-/- mice. Some of the mice injected with *in vitro* transdifferentiated BLaER1 cells developed tumors, and surprisingly, *ex vivo* residual tumors were found to transdifferentiate as well as control cells, suggesting that the C/EBPαER transgene becomes silenced in a subset of cells *in vivo*, permitting the cells' outgrowth into tumors. Mice injected with uninduced BLaER1 cells treated with tamoxifen pellets *in vivo* have shown a delay in tumor formation, but none of the animals injected remained tumor free. Even though we have shown that *in vitro* tamoxifen is able to promote transdifferentiation as well as E2, the route of administration of tamoxifen *in vivo* may not provide the concentration needed and may not guarantee the same level of the inducer in all the tissues. Experiments done by injecting different numbers of BLaER1 cells provided evidences that the number of injected cells is 50 times more than the number needed to induce the tumors. However, when we injected fewer

BLaER1 cells, we were able to show that C/EBPα is only slightly more effective in impairing tumor formation than with more cells. Data obtained by injecting RCH-ACV cells containing doxycycline inducible C/EBPα and treated with the drug have shown that none of the injected animals developed tumors, proving that transdifferntiated cells are stable and not tumorigenic. Furthermore, more than 50% of the animals injected with uninduced RCH-ACV cells and treated with doxycycline remain tumor free. However, the administration of doxycycline in the drinking water does not allow to control the intake dose of the inducer, a variable that may explain why some but not other animals develop tumors. The Burkitt lymphoma cell line Seraphina exhibits the t(8;14)(q24;q32) chromosomal translocation placing the *MYC* oncogene under the control of the *IGH* enhancer (Toujani et al., 2009). It therefore seemed possible that the observed C/EBPα-induced impairment of tumorigenicity is due to silencing of the *IGH* enhancer, as part of the extinction of the B-cell program, with the ensuing downregulation of *MYC*. However, this is unlikely to be the sole explanation since the tumorigenicity of the E2A-PBX transformed B-ALL cell line RCH-ACV was also impaired by C/EBPα, as was that of the Ras transformed murine pre-B-cell HAFTL (data not shown).

Our work differs from another study in which Eu-Myc murine B cell lymphomas were converted into myeloid cells by the v-Raf oncogene and the resulting myeloid cells retained tumorigenicity (Klinken et al., 1988). A possible difference between these two studies could be due to the dual role of C/EBPα in cell differentiation and cell cycle arrest. It is likely that the observed

C/EBPα induced quiescence during lymphoid cell transdifferentiation likely also contributed to the inhibition of tumor formation.

In summary, our data represent a proof of principle for the concept that transdifferentiation may constitute a novel therapeutic approach. To translate this potential approach to the clinic it would be essential to replace the transcription factor overexpression with small molecules or compounds (Zhou and Melton, 2008). To this end we performed a screening of around 2000 selected compounds by fluorescence microscope, using a myeloid gene reporter system based on the dsRed fluorescence (on/off). Due to their structure, C/EBPα and its partner PU.1 do not seem to be 'druggable' targets and so the library was chosen based on possible pathways involved in macrophage differentiation, available pharmacological and phytochemical compounds as well as molecules known to enhance iPS cell reprogramming. The BLaER1-LysRed system we developed turned out to be perfectly suitable for the screening, giving a simple and clear readout. However, the choice of generating the reporter line in the BLaER1 background (containing C/EBPαER-GFP), had the disadvantage to generate a number of 'false" hits, due to the presence in the libraries of ER agonist and analogues. Nevertheless, the presence of such molecules was used as an internal control in our screening. One alternative possibility would have been to generate a reporter line introducing the LysozymedsRed construct into the parental cell line Seraphina, or to generate a reporter system using the C/EBPα promoter fused to a florescent protein. This last approach, would have had the advantage of identifying compounds that directly activate

C/EBPα, and would have ruled out compounds that activate downstream C/EBPα targets, or even other transcription factors such as C/EBPβ, known to promote transdifferentiation into macrophages of murine B cells (Xie et al., 2004). In the validation experiments performed by FACS we noticed that the compounds treatment only affected the human BLaER1-LysRed cells and the mouse C11 cell line, both of which contain the inducible C/EBPα ER construct, while no effects were observed in the cell lines Seraphina, RCH-ACV and HAFTL, supporting the idea that these compounds activate the ER receptor, an thus the C/EBPα transgene. Moreover, the detection of increased granularity (SSC) of Seraphina and RCH-ACV cells treated with Coronaridine could be due to toxicity of the compound, also because it was not accompanied by an increase in size typically seen during transdifferentiation from B cells to macrophages. In conclusion, with this first screen we were not able to identify any compounds able to activate the endogenous macrophage program.

From the screen performed it seems unlikely that a single compound can mimic the effects of C/EBPα overexpression, but it remains possible that combinations of specific drugs would be active. For example, a compound that moderately activates C/EBPα could be effective in combination with another compound acting on the same pathway or in combination with drugs that modify chromatin, such histone acetylation. If drugs could be found that could induce the transdifferentiation of malignant B cells into quiescent macrophages at low concentrations they would be interesting for the therapy of

lymphoid neoplasms, especially when applied in combination with treatments that cause apoptosis.

Conclusions

1. Most of the leukemia and lymphoma cell lines tested can be induced by C/EBPα to transdifferentiate at least partially into macrophage-like cells.

2. Transdifferentiation induction depends on C/EBPα concentration.

3. Selected Burkitt lymphoma and B-ALL cell lines can be converted at essentially 100% efficiency into functional and quiescient macrophages.

4. Macrophages transdifferentiated from Burkitt lymphoma B cells retain their phenotype even when C/EBPα was inactivated, an hallmark of cell reprogramming.

5. C/EBPα activation *in vitro* and *in vivo* impaired the cells' tumorigenicity.

6. The BLaER1-LysRed reporter cell line we have generated is an efficient and robust reporter system for the screening of compounds able to convert malignant B cells into macrophages.

7. We have conducted a screen to identify compounds able to convert malignant B cells into macrophages, so far with negative outcome.

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