

C/EBP $\alpha$  poises B cells for rapid reprogramming into  
induced pluripotent stem cells

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*A mia moglie e mia madre*



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**ABSTRACT  
RESUMEN**

## THESIS ABSTRACT

One of the major goals of current stem cell research is understanding the mechanism of somatic cell reprogramming by Oct4, Sox2, Klf4 and Myc (OSKM) into induced pluripotent stem cells (iPSCs). However, the finding that only a small proportion of the cells become reprogrammed, typically requiring >12 days, has hampered progress towards this goal.

C/EBP $\alpha$  is a transcription factor specifically expressed in myelomonocytic cells within the hematopoietic system whose forced expression in B cells efficiently induces transdifferentiation into macrophages. We have now found that an 18-hour pulse of C/EBP $\alpha$  expression followed by OSKM activation induces an approximately 100-fold increase in the iPSC reprogramming efficiency, involving up to 95% of the cells within a week. Concomitantly, the cells undergo an epithelial-mesenchymal transition and pluripotency genes become upregulated to levels comparable to embryonic stem and iPS cells. In serum-free conditions the process is further accelerated, with 60% of the poised and OSKM induced B cells becoming Oct4-GFP positive within 2 days. These results are consistent with the idea that the C/EBP $\alpha$  pulse helps to overcome the stochastic phase of iPSC reprogramming. In addition, our work shed new light on the role of C/EBP $\alpha$  in induced pluripotency. Our data indicate that C/EBP $\alpha$  acts as a pathbreaker, at least in part mediated by the dioxygenase Tet2. C/EBP $\alpha$  binds to the Tet2 gene, induces its expression and translocates the protein to the nucleus. Here Tet2 binds to regulatory regions of pluripotency genes and converts methylated cytosine residues into hydroxymethylated cytosines. The pulse also renders the chromatin at regulatory sites of pluripotency genes accessible to DNase I digestion and, following OSKM induction, leads to local demethylation and to the binding of Oct4, correlating with the observed rapid upregulation of pluripotency genes. In line with an important role of Tet2 as a mediator of reprogramming, co-expression of the gene with OSKM enhanced B cell reprogramming substantially. The rapid and highly efficient iPSC reprogramming approach described herein should help to fully elucidate the early events of



reprogramming to pluripotency and, if applicable to human cells, could have potential clinical applications.

## RESUMEN DE TESIS

Actualmente uno de los principales objetivos de la investigación con células madre es la comprensión de los mecanismos por los cuales las células somáticas se pueden reprogramar a células madre pluripotentes inducidas (iPSCs) por la acción de los factores de transcripción Oct4, Sox2, Klf4 y Myc (OSKM). Sin embargo, la baja eficiencia de este proceso, que tiene lugar sólo en un pequeño porcentaje de células y que típicamente requiere más de 12 días para llevarse a cabo, ha impedido la consecución de grandes avances en este campo en los últimos años. C/EBP $\alpha$  es un factor de transcripción específico de células del linaje mielo-monocítico del sistema hematopoyético. La expresión ectópica de esta proteína en células B puede inducir su transdiferenciación a macrófagos. En nuestro estudio de investigación hemos descubierto que la exposición de C/EBP $\alpha$  durante 18 horas seguida de la activación de OSKM, aumenta en 100 veces la eficiencia de reprogramación de las iPSC, resultando en la reprogramación del 95% de las células después de una semana. En detalle, durante este proceso de reprogramación las células experimentan una transición epitelio-mesénquima y los genes de pluripotencia se expresan en niveles comparables a los expresados en células madre embrionarias y iPSC. Cuando la reprogramación se lleva a cabo en medio de cultivo sin suero el proceso es aún más rápido, de tal modo que el 60% de las células B inducidas por C/EBP $\alpha$  y OSKM son positivas para Oct4-GFP en tan sólo dos días. Estos resultados apoyan la idea de que una exposición transitoria de C/EBP $\alpha$  ayuda a superar la fase estocástica de la reprogramación de las iPSC. Además, nuestros descubrimientos aclaran el papel de C/EBP $\alpha$  en el proceso de pluripotencia inducida, indicando que actúa como un catalizador, mediado en parte por la actividad de la dioxigenasa Tet2. De tal modo, que C/EBP $\alpha$  se une a regiones reguladoras del locus de Tet2, induciendo de esta manera su expresión y translocando la proteína al núcleo. Una vez en el núcleo, Tet2 se une a las regiones regulatorias de los genes de pluripotencia y convierte los residuos de citosinas metilados existentes en estas regiones en citosinas hidroximetiladas. Además, la exposición transitoria de C/EBP $\alpha$

deja la cromatina más accesible a la digestión con DNasa I alrededor de las regiones regulatorias de los genes de pluripotencia y, tras la inducción con OSKM, desencadena una demetilación local favoreciendo la posterior unión de Oct4 a estas regiones. Todo ello finalmente promueve la expresión concomitante de los genes de pluripotencia. Adicionalmente, en nuestro estudio se demuestra que la coexpresión de Tet2 y OSKM aumenta significativamente la reprogramación de las células B, lo cual se encuentra en línea con un papel importante de Tet2 en la reprogramación. En resumen, en este estudio se presenta el sistema de reprogramación de iPSC más rápido y eficiente descrito a día de hoy. El cual, facilitará la comprensión de los eventos precoces en el proceso de reprogramación a pluripotencia y, en el caso de que se pueda extrapolar a células humanas, podrá tener aplicaciones clínicas relevantes en el campo de la medicina regenerativa.

## **PREFACE**

The work presented in this doctoral thesis was supported by the Ministerio de Educacion y Ciencia and was carried out in the laboratory of hematopoietic stem cells, transdifferentiation and reprogramming at the Centre for Genomic Regulation (CRG) in Barcelona, Spain under the supervision of Prof. Dr. Thomas Graf (ICREA, CRG).

The content of this thesis provides novel insights in the mechanism of reprogramming of somatic cells to induced pluripotent stem cells and was published in Nature 2014, 506 (235–239).

## **PART I**

### **INTRODUCTION AND AIMS**

## **Introduction**

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## 1. ON THE ROAD TO IPS CELLS

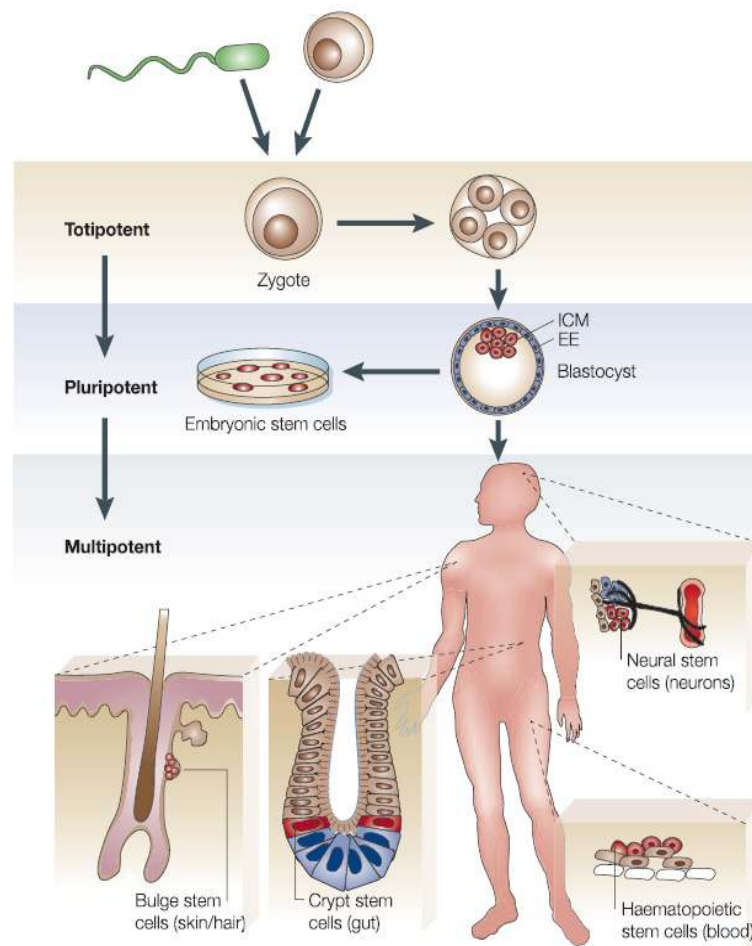
All cell types that exist in the body, including somatic cells of all three germ layers as well as germ cells originate from pluripotent stem cells of the embryo. The recently developed induced pluripotent stem cell (iPSC) technology (Takahashi and Yamanaka, 2006), which enables the induction of pluripotency in mature somatic cells by treatment with defined factors, has created new avenues in basic research, disease modeling and regenerative medicine. This discovery represents the synthesis of scientific principles and technologies that have been developed over the past six decades, i.e. the observations that somatic cells retain certain plasticity; the derivation and maintenance in culture of pluripotent stem cells and, finally, the observation that transcription factors are key determinant of cell fate whose enforced expression can switch one mature cell type into another.

### 1.1 Changing cellular potential

During mammalian development, cells gradually lose potential and become progressively differentiated to fulfill the specialized functions of somatic tissues (Fig. 1). Only zygotes and blastomeres of early morulas retain the ability to give rise to all embryonic and extraembryonic tissues (Kelly, 1977), and are therefore called “totipotent”. In contrast, cells of the inner cell mass (ICM) of the blastocyst (from which embryonic stem (ES) cell lines are derived (Evans and Kaufman, 1981; Martin, 1981)) can give rise to all embryonic but not all extraembryonic tissues, and are hence called “pluripotent”. Finally, adult stem cells can only give rise to cell types within specific tissues (blood, neurons, muscle etc.) and are called either “multipotent”, bipotent or ‘monopotent,’ depending on the developmental options they have. Upon terminal differentiation, cells entirely lose their developmental potential (Stadtfeld and Hochedlinger, 2010).

For a long time, differentiation process was considered to be a one-way street, with cell states flowing along the valleys within the ‘epigenetic landscape’

proposed by Conrad Waddington (Waddington, 1957). In parallel, it was also believed that unnecessary genetic information becomes deleted in cells committed to a specific state, a theory known as Weismann's barrier (Weismann, 1893).



**Figure 1. The stem cell hierarchy.** The totipotent zygote formed by the fusion of egg and sperm divides to form the inner cell mass (ICM) and the extra-embryonic (EE) tissue of the blastocyst. When isolated from the blastocyst *in vitro*, the cells of the ICM can be maintained in culture as pluripotent embryonic stem cell (ESC) lines. During the development of the embryo, the pluripotent stem cells in the ICM become increasingly restricted in their lineage potential and generate tissue-specific, multipotent stem cells. Adapted from Eckfeldt et al., 2005.

Subsequent classic studies, however, not only suggested that 'committed' cells retain all genetic information, but that they can also change their fate in response to specific stimuli. In one of these studies, cells from the imaginal discs of *Drosophila melanogaster* pupae were serially transplanted into the abdomen of an adult fly, and 'transdetermination' was observed: cells that were



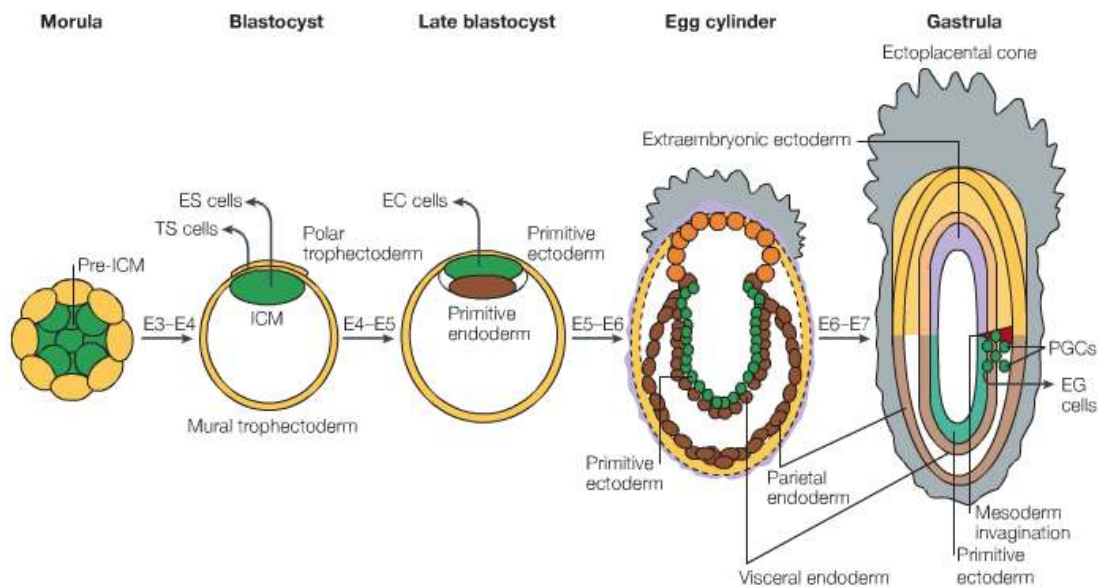
originally destined to form genital structures gave rise to leg or head structures and, eventually, on subsequent transplantations, to wings (Gehring, 1967; Hadorn, 1966). Although such switches in cell fate occurred at low frequency, these experiments by Hadorn and Gehring provided evidence that explanted cells are surprisingly plastic. In another elegant study (Le Lievre and Le Douarin, 1975), cells were transplanted from quails to chickens: these cells were sufficiently similar to be able to participate in normal development after transplantation but exhibited histologically distinguishable nuclei, enabling them to be tracked. Using this property, Le Lievre and Le Douarin (Le Lievre and Le Douarin, 1975) showed that neural crest cells transplanted into a new location can adopt new fates (bone, cartilage and connective tissue) dictated by their new cellular neighborhood in the avian embryo.

Parallel experiments, conducted in a number of different species, showed that transfer of nuclei from both embryonic and adult somatic cell types into enucleated oocytes can lead to the formation of all three germ layers and even to the generation of entire new animals (see chapters 1.3 and 2) (Gurdon, 2013; Gurdon and Byrne, 2004; Gurdon and Melton, 2008; Hochedlinger and Jaenisch, 2002; Wilmut et al., 1997), unequivocally demonstrating that the identity of differentiated cells can be fully reversed.

## **1.2 Pluripotent cell lines**

A major discovery towards the isolation of iPS cells was the establishment of embryonic carcinoma cells (ECCs) from tumors of germ cell origin (Fig. 2) (Kleinsmith and Pierce, 1964; Stevens and Little, 1954). ECCs could be cultured indefinitely while retaining pluripotency and differentiation potential (Finch and Ephrussi, 1967; Kahan and Ephrussi, 1970). Importantly, when ECCs were fused with somatic cells, such as thymocytes, the resulting hybrids acquired the developmental properties of ECCs, extinguishing the features of the somatic fusion partner (Miller and Ruddle, 1976, 1977). The preeminence of the pluripotent state over the somatic program in hybrids suggested that soluble factors must exist in ECCs that can confer a pluripotent state in somatic cells,

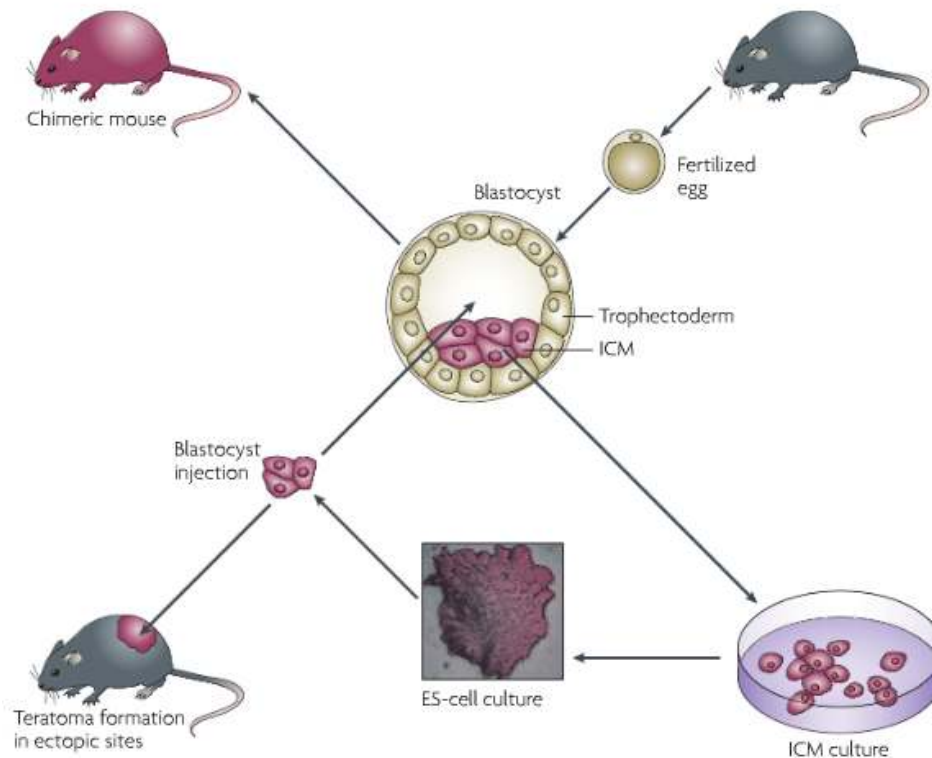
and that these factors should be identifiable. Unfortunately, most ECC lines are aneuploidy and contributed poorly to adult somatic cell tissue (Brinster, 1974) and rarely to germline upon injection into blastocysts (Bradley et al., 1984; Stewart and Mintz, 1982), a key assay to probe pluripotency of nonhuman cells (Gardner, 1968).



**Figure 2. Pluripotent cells in the embryo.** Pluripotent cells of the embryo are tracked in green. From left to right, the morula-stage mouse embryo (embryonic day 2.5; E2.5) holds a core of pre-ICM (inner cell mass) cells that turn into ICM cells at blastula formation (E3.5). At this stage, embryonic stem cell (ESC) and trophoblast stem cell (TSC) lines can be derived *in vitro*, and implantation occurs *in vivo*. At E5 pluripotent cell lines known as embryonic carcinoma cells (ECCs) can be derived from the primitive ectoderm. At E6 and subsequent stages, the experimental ability to derive ESCs, TSCs and ECCs from the mouse embryo is progressively lost, and the embryo starts gastrulating. This process involves the formation of a mesoderm layer between ectoderm and endoderm, and the formation of the primordial germ cells (PGCs). Pluripotent cell lines can be derived from later germ-cell stages, namely embryonic germ cells (EGCs) from PGCs, and germline stem cells (GSCs) from neonatal testis. Adapted from Boiani and Scholer, 2005.

The finding that ECCs can also be derived from teratocarcinomas experimentally induced by subcutaneous transplantation of implantation-stage mouse embryos into histocompatible hosts motivated attempts to isolate pluripotent cells directly from such embryos. These efforts succeeded many years later, leading to the derivation of embryonic stem cells (ESCs) from the ICM of mouse blastocysts (Fig. 3) (Evans and Kaufman, 1981; Martin, 1981) and, subsequently, also from human embryos (Thomson et al., 1998). ESCs are karyotypically normal and contribute efficiently to all adult tissues including

the germline. In fact, ESCs are able to produce entire animals after injection in tetraploid blastocyst (Eggan et al., 2001; Nagy et al., 1990).



**Figure 3. Derivation of embryonic stem cells.** ICM cells cultured under appropriate conditions give rise to cell lines known as embryonic stem cells (ESCs). These cells integrate into the embryo after injection in blastocysts and give rise to all cell lineages in the body (chimeric mice), including germ cells. When ESCs are injected subcutaneously they generate teratomas. Adapted from Nishikawa et al., 2007.

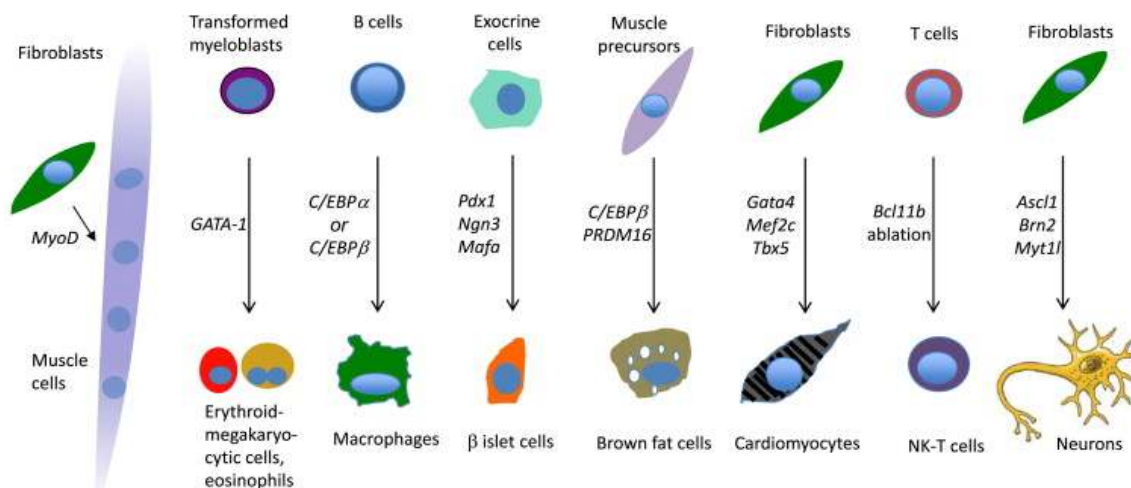
Pluripotent cell lines have also been derived from other embryonic and adult tissues upon explantation in culture (Fig. 2). For example: epiblast-derived stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007) have been isolated from post-implantation embryos, embryonic germ cells (EGCs) (Matsui et al., 1992; Resnick et al., 1992) have been derived from primordial germ cells (PGCs) of the mid-gestation embryo, and multipotent germline stem cells (mGSCs) have been generated from explanted neonatal (Kanatsu-Shinohara et al., 2004) and adult (Guan et al., 2006; Ko et al., 2009; Seandel et al., 2007) mouse testicular cells. While ESCs, ECCs, mGSCs, and EGCs are pluripotent, only ESCs are able to generate embryos in the tetraploid aggregation assay. This is because

ESCs carry balanced parental imprints that are critical for normal development, whereas EGCs and mGSCs have erased imprints or paternal-only imprints, respectively, as a result of germline development (Hochedlinger and Jaenisch, 2006). However, all of the pluripotent cell lines tested so far (ESCs, ECCs, and EGCs) have been shown to induce pluripotency in somatic cells after cellular fusion, demonstrating that they have dominant transacting factors (Cowan et al., 2005; Tada et al., 1997; Tada et al., 2001). The undifferentiated pluripotent cell state is dependent on the expression of a combination of transcription factors, most notably Oct4, Sox2, and Nanog, and by external signaling through the cytokines leukemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4) (Rossant, 2008; Ying et al., 2003). Co-regulatory and auto-regulatory mechanisms appear to link Oct4, Sox2, and Nanog in a recursive self-reinforcing circuit. Each factor is essential for the pluripotent cells in the blastocyst, whereas deletions from ES cells provoke cell differentiation (Niwa, 2007). Recently it has been found that blockage of mitogen-activated protein kinase (MAPK) and suppression of glycogen synthase kinase-3 (Gsk3) with selective small molecule inhibitors (2i) is sufficient to stabilize and sustain ESCs with full pluripotency (Silva and Smith, 2008; Ying et al., 2008).

### **1.3 Transcription factors in lineage switching**

The third principle that contributed to the discovery of induced pluripotency was the observation that lineage-associated transcription factors, which help to establish and maintain cellular identity during development by driving the expression of cell type-specific genes while suppressing lineage-inappropriate genes, can change cell fate when ectopically expressed in certain heterologous cells (Fig. 4) (Graf, 2011; Graf and Enver, 2009). Lineage conversion was first described in 1986 by Lassar and colleagues, who switched fibroblasts to a myoblast-like fate by exposure to the demethylating agent 5-azacytidine and the ectopic expression of cDNAs (Lassar et al., 1986). Subsequently, Davis and colleagues identified MyoD as the transcription factor driving this conversion (Davis et al., 1987). These initial experiments demonstrated how transcription

factors could switch the fate of a differentiated somatic cell into another phenotype. Fundamental insights into the instructive roles of transcription factors in lineage specification came from studies in the hematopoietic system, which is probably the best-defined cellular differentiation system in mammals (Graf and Enver, 2009; Orkin and Zon, 2008).



**Figure 4. Examples of transcription factor-induced transdifferentiation.** Adapted from Graf, 2011.

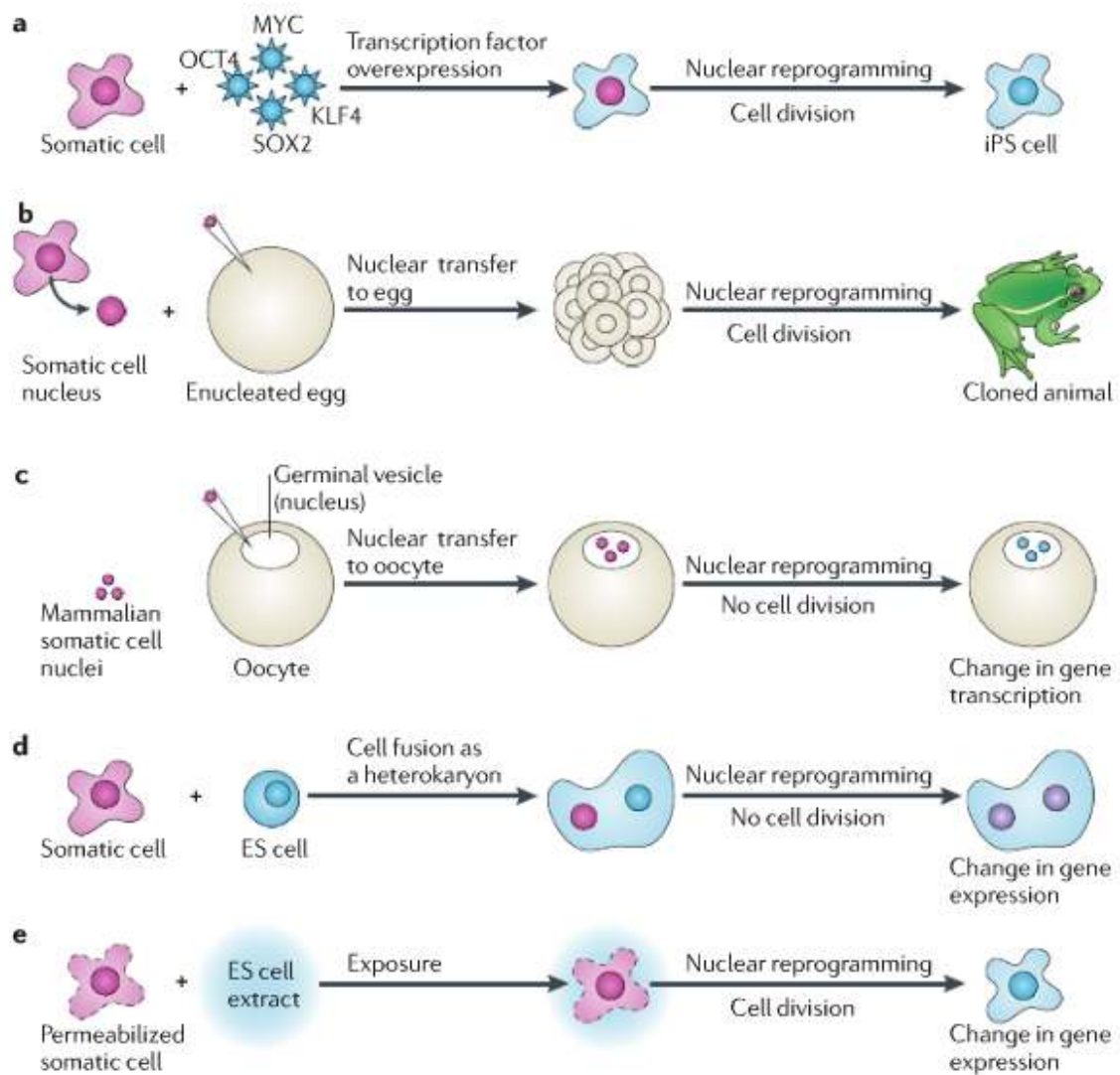
Lineage conversion experiments showed that forced expression of GATA1 was sufficient to induce erythroid and megakaryocytic markers in monocytic cell lines (Kulesa et al., 1995). These experiments suggested that transcription factors not only activate novel gene expression programs but also repress the programs specific to the starting cell, a hallmark of transdifferentiation. Subsequently, it was found that forced expression of PU.1 in megakaryocytic and erythrocyte precursors converts these cells into myeloblasts (Nerlov and Graf, 1998). However, these experiments did not address the question of whether mature hematopoietic cells were equally plastic. The answer arrived in 2003, when Graf and colleagues discovered that primary B and T cells could be converted very efficiently into functional macrophages upon overexpression of the myeloid transcription factor C/EBP $\alpha$  (see also chapter 4) (Laiosa et al., 2006; Xie et al., 2004). Further work demonstrated that the combination of C/EBP $\alpha$  and PU.1 is sufficient to induce macrophage-like cells from primary and NIH3T3 fibroblasts (Feng et al., 2008). A breakthrough in the

transdifferentiation field was the discovery that insulin-producing cells in the pancreas could be obtained by *in vivo* transdifferentiation (Zhou et al., 2008). These authors screened 1,100 transcription factors by *in situ* hybridization in the pancreas and identified 20 that are expressed in mature  $\beta$  cells and their precursors, of which 9 led to  $\beta$  cell phenotypes when mutated. The adenoviral mediated overexpression of these 9 factors into the pancreas of immunodeficient mice, increased the number of  $\beta$  cells, an effect that was found to be mediated by *Ngn3*, *Pdx1*, and *Mafa*. Lineage-tracing experiments showed that these three factors converted >20% of endocrine cells into cells closely resembling  $\beta$  cells. Remarkably, by using a mouse model for diabetes type one (Zhou et al., 2008), Melton and colleagues showed that the *in vivo* transdifferentiation alleviated the hyperglycemia caused by insulin deficiency. More recently, Kajimura and colleagues identified C/EBP $\beta$  as a partner of PRDM16 and showed that these two transcription factors can induce a highly efficient switch from mouse and human fibroblast into brown fat cells (Kajimura et al., 2009). Another example of transdifferentiation is the conversion of fibroblast in cardiomyocytes with a combination of *Gata4*, *Mef2c*, and *Tbx5* (Ieda et al., 2010; Qian et al., 2012). In 2010 Wernig and colleagues showed direct conversion of fibroblasts into induced neuron-like cells by the activation of the neural factors *Ascl1*, *Brn2*, and *Myt1l* (Vierbuchen et al., 2010). About 1 year later, this approach was translated to human fibroblasts (Ambasudhan et al., 2011; Pang et al., 2011; Vierbuchen et al., 2010). Remarkably, expression of miR-9/9\* together with miR-124 was shown to convert fibroblasts into neuron-like cells even without overexpression of transcription factors (Yoo et al., 2011). Different subtypes of neurons have also been obtained by lineage conversion from mouse and human fibroblasts (Caiazzo et al., 2011; Son et al., 2011). Recently, induced neural precursor cells were generated from a mesoderm derived population by using lineage specific transcription factors (Han et al., 2012; Lujan et al., 2012; Ring et al., 2012; Sheng et al., 2012). Of note, these experiments proved that lineage conversions can be achieved not only between cell types within the same tissue or even germ layer, since fibroblasts are of mesodermal origin, whereas neurons are derived from

ectoderm. Some of the early transdifferentiation experiments provided the intellectual framework for a more systematic search for transcription factors that could induce the conversion of differentiated cells to a pluripotent state.

## 2. REPROGRAMMING TO PLURIPOTENCY

Besides transcription factor mediated cell reprogramming, several different strategies such as nuclear transfer to eggs or oocytes, cell fusion and extract treatment have been employed to induce the conversion of differentiated cells into an embryonic state (Fig. 5).





**Figure 5. Different experimental approaches to nuclear reprogramming.** (a) induced pluripotency. The expression of four transcription factors (Krüppel-like factor 4 (KLF4), MYC, OCT4 and Sry-box containing 2 (SOX2)) can reprogram somatic cells to a state that is similar to that of embryonic stem (ES) cells, and these cells are called induced pluripotent stem (iPS) cells. (b) Nuclear transfer to eggs. A single *Xenopus laevis* (or mammalian) somatic cell nucleus) when transplanted into an enucleated *X. laevis* (or mammalian) egg can give rise to an entire new animal. (c) Nuclear transfer to oocytes. In these experimental conditions, the nucleus does not undergo cell division and does not generate new cell types. However, it undergoes direct reprogramming in that it expresses pluripotency genes. (d) Cell fusion. A differentiated cell is fused to another cell, such as an ES cell. In the resulting heterokaryon, the nucleus of the differentiated cell is reprogrammed to express pluripotency genes. (e) Reversible permeabilization and exposure to ES cell extract. Somatic cells permeabilized with streptolysin O briefly exposed to ES cell extract start expressing pluripotency genes at low levels. Adapted from Jullien et al., 2011.

## 2.1 Nuclear transfer

Nuclear transfer involves the physical transplantation of a single nucleus into a meiotic metaphase II arrested egg, usually after removing the recipient egg's genetic material. It was by this method that the first cloning of amphibians and later of mammals was performed (Gurdon et al., 1958; Wakayama et al., 1998; Wilmut et al., 1997) and it is often called 'somatic cell nuclear transfer' (SCNT). Nuclear transfer has been successfully used to reprogram many different species, including human cells (Byrne et al., 2007; Cibelli, 2007; Noggle et al., 2011; Tachibana et al., 2013; Wakayama and Yanagimachi, 1999) and much of our understanding of reprogramming comes from experiments using this system (Gurdon and Wilmut, 2011). The cloned embryonic cells resulting from this procedure may also be cultured *in vitro*, giving rise to embryonic stem cell lines (Byrne et al., 2007; Noggle et al., 2011; Tachibana et al., 2013). Nuclear reprogramming by this route is thought to 'mimic' natural fertilization (Gurdon, 2013). Reprogramming by SCNT is mediated by natural components of the egg and involves extensive DNA replication and cell division (Jullien et al., 2011). The efficiency of the process, as attested by the generation of entirely normal adult animals, is low, below ~1-2% when adult somatic nuclei are used as donors (Yang et al., 2007). Additionally, some clones may display a number of abnormalities, phenotypically and at both a molecular and physiological level (Wilmut et al., 1997; Yang et al., 2007). The low efficiency and abnormalities are likely to be attributed to a failure to completely reprogram the donor genome.

This is often called ‘epigenetic memory’, in which a memory of the donor cell gene expression is retained by some cells of the resulting cloned embryos. This has been shown to be in part due to incomplete resetting of the epigenome, such as DNA methylation and histone variants, in the donor nucleus (Ng and Gurdon, 2008).

## **2.2 Reprogramming by cell fusion**

Epigenetic reprogramming of somatic nuclei to an undifferentiated state has been demonstrated in murine hybrids produced by fusion of embryonic cells with somatic cells. Upon cell fusion, the nuclei of both cell types can either remain separated in the common cytoplasm (heterokaryon formation) or, after mitosis, fuse to form a hybrid genome (synkaryon). As mentioned in the previous chapter hybrids between various somatic cells and embryonic carcinoma cells (Solter, 2006), embryonic germ, or ES cells (Zwaka and Thomson, 2005) share many features with the parental pluripotent cells, indicating that this phenotype is dominant in such fusion products. As with mouse (Tada et al., 2001), human ES cells have the potential to reprogram somatic nuclei after fusion (Cowan et al., 2005; Yu et al., 2006). Activation of silent pluripotency markers such as *Oct4* or reactivation of the inactive somatic X chromosome provided molecular evidence for reprogramming of the somatic genome in the hybrid cells (Jaenisch and Young, 2008). It has been suggested that DNA replication is essential for the activation of pluripotency markers during cell fusion (Fisher) which is first observed 2 days after fusion (Do and Scholer, 2004). In addition, forced overexpression of *Nanog* in ES cells promotes pluripotency when fused with neural stem cells (Silva et al., 2006). Heterokaryon mediated reprogramming may also work with cells from different species (Yamanaka and Blau, 2010). The latter case permits researchers to easily follow transcriptional changes in the somatic nucleus without contributing signals from ‘carry-over’ transcript derived from the dominant cell. Moreover, the ability to manipulate both the responding and dominant cells using standard cell culture techniques makes cell fusion a useful technique for studying the

mechanisms that underpin the early stages of reprogramming (Piccolo et al., 2011). However, the fact that the cells reprogrammed after fusion are tetraploid presents a major shortcoming of this approach for applications in cell therapy.

### **2.3 Reprogramming by extract treatment**

Reprogramming may also partly be achieved by exposing permeabilized cells to protein extracts that are prepared from pluripotent ES cells (Taranger et al., 2005). Permeabilized cells re-sealed after treatment with the extract and then cultured, activate previously silent pluripotency genes. This reprogramming is thought to be associated with cell division and DNA replication in most instances. However, the efficiency of extract-based reprogramming is very low (Bru et al., 2008). An appealing aspect of reprogramming by cell extract is the ability to deplete candidate reprogramming factors from the extract and test their activity. Furthermore, the use of extract provides an opportunity for biochemical fractionation, potentially allowing investigators to identify novel reprogramming factors (Singhal et al., 2010), although reproducibility of extract treatments is a concern in the field (Liu et al., 2011; Singhal et al., 2010). This is probably due to the difficulty of preparing high quality extracts, which might be solved with further developing of this system.

### **2.4 Generation of iPS cells by defined transcription factors**

In order to identify the transcriptional regulators that can reprogram somatic cells into pluripotent stem cells, Yamanaka and Takahashi designed an elegant screen for factors within a pool of 24 pluripotency-associated candidate genes based on the activation of the ES specific gene *Fbx15* (Takahashi and Yamanaka, 2006). The combination of the 24 factors indeed generated ES-like colonies in which the *Fbx15* locus was activated. They then used a reductive or “leave one out” strategy to determine the minimal set of factors required to reprogram fibroblasts into pluripotent-like cells. Thus, the reprogramming cocktail of Oct4, Sox2, Klf4, and Myc was defined (the so called Yamanaka

factors or, simply, OSKM). The cells obtained were shown to express the pluripotency markers *Nanog* and *SSEA-1* and to generate teratomas when injected subcutaneously in immunocompromised mice (Takahashi and Yamanaka, 2006). However, these “first generation” iPS cells were only partially reprogrammed as they expressed lower levels of several key pluripotency genes compared with ES cells, showed incomplete demethylation of the *Oct4* promoter and failed to generate live chimeras (Takahashi and Yamanaka, 2006). When activation of endogenous *Nanog* or *Oct4* was used as a more stringent selection criterion for pluripotency, the resulting Oct4-iPS or Nanog-iPS cells, in contrast to the Fbx15-iPS cells, were fully reprogrammed to a pluripotent ES cell state by molecular and biological criteria (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). These “second generation” iPS cells showed: (1) global gene expression and chromatin configuration identical to ES cells; (2) complete demethylation of *Nanog* and *Oct4* loci; (3) reactivation of X-chromosome in female lines (Maherali et al., 2007); (4) contribution to germline-competent chimeras; (5) correct expression of all well-known pluripotency markers. Finally, by using inducible lentiviral vectors it was shown that the four factors need to be expressed for at least 12 days to obtain iPS cells (Brambrink et al., 2008; Stadtfeld et al., 2008b). These experiments also suggested that the frequency of reprogramming increased with time, resulting in up to 0.5% of the input MEFs giving rise to iPS cells at 3 to 4 weeks after infection (Meissner et al., 2007). More recently, iPS cell lines capable of generating “all-iPS” mice upon injection into tetraploid blastocyst have been described (Boland et al., 2009; Kang et al., 2009; Stadtfeld et al., 2010a). iPS cells have also been derived from a number of different species, including humans (Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007), rats (Li et al., 2009b), rhesus monkeys (Liu et al., 2008) and from some endangered species (Ben-Nun et al., 2011) demonstrating that fundamental features of the pluripotency transcriptional network are conserved during evolution. Similarly, iPS cells have been derived from other somatic cell types, such as keratinocytes (Aasen et al., 2008), neural cells (Di Stefano et al., 2009; Kim et al., 2008), stomach and liver cells (Aoi et al., 2008), melanocytes (Utikal et al.,

2009a), pancreatic cells (Stadtfield et al., 2008a) and hematopoietic cells (Eminli et al., 2009; Hanna et al., 2008), further underscoring the universality of induced pluripotency. Of note, stem cells or certain progenitor cells seem to be more readily reprogrammed to become iPS cells, probably due to their expression of a subset of pluripotent stem cell genes (Di Stefano et al., 2009; Yamanaka and Blau, 2010).

### **2.4.1. Technical advances in iPS cell generation**

Major problems need to be overcome in order to use iPS cells as an efficient research tool and to translate this technology to the clinic, i.e. the genetic modification of the target somatic cells and the low efficiency of reprogramming. Recent efforts are moving towards this direction.

*Factors that can enhance reprogramming or substitute known reprogramming factors*

The four reprogramming factors initially identified can be complemented or substituted with different factors. Many of these factors are genes normally expressed during early development and in cultured pluripotent cells. This is the case of *Nanog*, that when overexpressed in B cells reduces the time for appearance of iPS cell colonies by half (Hanna et al., 2009). Another example is the pluripotency transcription factor *UTF1*, that when overexpressed in human fibroblasts in combination with the Yamanaka factors, increases the number of iPS cell colonies significantly (Zhao et al., 2008). Furthermore, the individual expression of *Sall4*, *Esrrb* and *Zfp296* in combination with OSK increments the overall number of reprogrammed colonies (Feng et al., 2009; Fischedick et al., 2012; Tsubooka et al., 2009). Likewise, addition of *Tbx3* in reprogramming factors seemed to improve the quality of iPS cells (Han et al., 2010). Lin28, a negative regulator of the *let7* microRNA (miRNA) family, gradually decreases during ES cell differentiation (Di Stefano et al., 2011) and has been shown to accelerate the efficiency of iPS cell generation in a cell

cycle-dependent manner (Hanna et al., 2009). Another Lin-related factor, Lin41, has been recently implicated in enhancing the efficiency of human iPS reprogramming (Worringer et al., 2014). In an elegant study, Yamanaka and colleagues screened by overexpression 33,275 factors identifying Glis1, a transcription factors expressed in the oocyte but not in the blastocyst nor in the ES cells, as capable of enhancing iPS efficiency of mouse and human cells. Glis1 might represent a link between reprogramming during iPSC generation and reprogramming after nuclear transfer.

Cell cycle related genes have been also used in iPS reprogramming to modulate the efficiency of the process. Telomerase reverse transcriptase (TERT) and SV40 large T antigen, two proteins that have positive effect on cell proliferation, increase the appearance of iPS cell colonies when used in combination with OSKM (Park et al., 2008). In addition removal of cell-cycle control checkpoints by disruption of the signaling pathways mediated by the tumour-suppressor protein p53 or the cell-cycle regulator INK4A result in an accelerated formation of iPSCs (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009a; Marion et al., 2009; Utikal et al., 2009b). MicroRNAs (miRNAs) are also known to influence pluripotency and reprogramming (Leonardo et al., 2012). Some miRNAs from the mir290 cluster contribute to the unique cell cycle of the ES cells (Wang et al., 2008). The introduction of miR-291-3p, miR-294 or miR-295 enhances iPS reprogramming efficiency by modulating Myc expression (Judson et al., 2009). miRNAs have also been shown to promote the mesenchymal to epithelial transition (MET), including miR-205, the miR-200, miR-302 and -miR-372 families (Gregory et al., 2008; Leonardo et al., 2012). There is also indirect evidence that miRNAs regulate the epigenetic state in iPSCs. The miR-302 and miR-372 in human fibroblasts regulate the expression of the DNA-binding protein genes methyl-CpG binding protein 2 (*MECP2*) and methyl-CpG binding domain protein 2 (*MBD2*) (Subramanyam et al., 2011). On the other hand, there is evidence that the miR-29 family members directly regulate both *DNMT3A* and *DNMT3B* RNAs (Fabbri et al., 2007).

### *Culture conditions and chemical compounds that enhance reprogramming*

Reprogramming under hypoxic conditions of 5% O<sub>2</sub> (similar to the pH found in some stem cell niches), instead of the atmospheric 21% O<sub>2</sub>, increases the reprogramming efficiency of mouse and human cells 4-40 fold. When combined with valproic acid, an inhibitor of histone deacetylases, the efficiency increases to 200-fold in mouse cells (Yoshida et al., 2009). The feeder cells used as support and the addition of certain cytokines have been also reported to influence reprogramming efficiency. For instance Wnt3a promotes the generation of iPS cells in the absence of Myc (Lluis et al., 2008; Marson et al., 2008).

Most small molecules that enhance somatic cell reprogramming are able to compensate for three of the four canonical factors, SKM (Federation et al., 2013). The most widely used compounds are VitaminC, Valproic acid and Azacytidine (Esteban et al., 2010; Huangfu et al., 2008; Stadtfeld et al., 2012). The use of 2i in cell reprogramming has been reported to increase the number of fully reprogrammed clones from neural stem cells (Silva et al., 2008). Earlier this year, the first successful reprogramming experiment using only small compounds was published (Hou et al., 2013), using a combination of VPA, CHIR99021, 616452 (an anaplastic lymphoma kinase (ALK) inhibitor), tranylcypromine (an LSD1 inhibitor), forskolin (an adenylyl cyclase activator), and late treatment with the global methylation inhibitor DZNep, which leads to broad reduction in histone methylation. Although the yield of iPSC colonies was exceedingly low these findings are very exciting for their possible clinical application. It will be interesting to understand the mechanisms underlying the activity of these new molecules.

### *Transcription factor delivery systems*

A number of different approaches have been used to shuttle reprogramming factors into somatic cells, and these can affect reprogramming efficiency. The delivery of OSKM factors in mouse and human cells was originally achieved

using a Moloney murine leukaemia virus (MMLV)-derived retrovirus (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). While retroviral transgene are usually silenced toward the end of the reprogramming due to the activation of both DNA and histone methyltransferases (Lei et al., 1996; Matsui et al., 2010), this process is often incomplete, resulting in partially reprogrammed cell lines that continue to depend on exogenous factor expression and fail to activate the corresponding endogenous genes (Mikkelsen et al., 2008; Takahashi and Yamanaka, 2006). In addition residual retroviral expression or reactivation of the exogenous factor can impair the developmental potential of the iPS cell lines (Takahashi and Yamanaka, 2006) and frequently induces tumor formation in chimeric mice (Okita et al., 2007). The efficiency of IPS cell generation using retroviral vectors expressing the OSKM genes separately is ~0.1% in MEFs and ~0.01% in human fibroblasts (Gonzalez et al., 2011). HIV-derived lentiviral vectors have also been used to express different sets of transcription factors in somatic cells (Blelloch et al., 2007; Yu et al., 2007). The efficiency of reprogramming using lentiviral vectors is comparable to that of retroviruses and have the advantage that they can be used to infect non-dividing cells and, because of their packaging capacity, offer the opportunity to express polycistronic cassettes encoding all four reprogramming factors (Carey et al., 2009; Sommer et al., 2009). The use of inducible lentiviral vectors whose expression can be controlled by doxycycline allows for the selection of fully reprogrammed, transgene-independent iPSCs (Brambrink et al., 2008; Stadtfeld et al., 2008b). Inducible vector systems have also been employed to generate so-called “secondary” reprogramming systems, which do not rely on direct factor delivery into target cells. These systems use differentiating “primary” iPSC clones, generated with doxycycline-inducible lentiviral vectors, into genetically homogeneous somatic cells using either *in vitro* differentiation or blastocyst injection (Hockemeyer et al., 2008; Maherali and Hochedlinger, 2008; Wernig et al., 2008a). These somatic cells are then cultured in doxycycline-containing media, thus causing the formation of “secondary” iPSCs at efficiencies that depend on the specific cell type used but are generally several orders of magnitude higher than the efficiencies obtained after primary



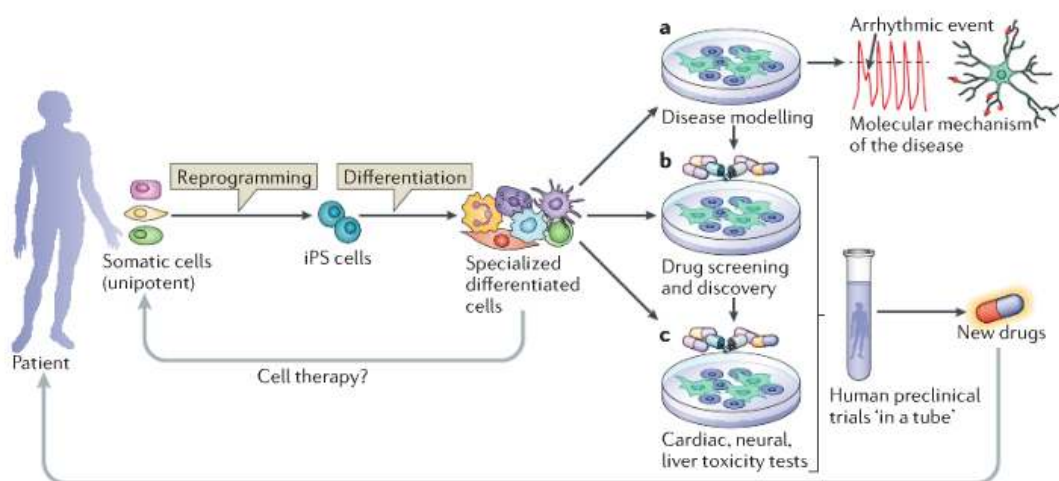
infection (Wernig et al., 2008a). Recently, “reprogrammable” mouse strains have been developed that contain a single inducible polycistronic transgene in a defined genomic position, as well as the tetracycline-controlled transactivator (rtta) (Carey et al., 2010; Stadtfeld et al., 2010b).

Approaches to derive iPSCs free of transgenic sequences are aimed at circumventing the potentially harmful effects of leaky transgene expression and insertional mutagenesis. This is particularly important when considering iPSC technology in a therapeutic setting. The first integration-free iPSCs were generated from adult mouse hepatocytes using non-integrating adenoviral vectors (Stadtfeld et al., 2008c) and from MEFs transfected with plasmids (Okita et al., 2008). These experiments provided the proof of principle that transient expression of the four classical reprogramming factors is indeed sufficient to induce pluripotency in somatic cells. Human fibroblasts have also been reprogrammed into iPSCs with adenoviral vectors (Zhou and Freed, 2009) and Sendai virus (Fusaki et al., 2009), as well as with polycistronic mini-circle vectors (Jia et al., 2010) and self-replicating selectable episomes (Yu et al., 2009). Reprogramming efficiencies with current non-integrating methods are several orders of magnitude lower (~0.001%) than those achieved with integrating vectors, most likely because factor expression is not maintained for a sufficient length of time to allow complete epigenetic remodeling (Stadtfeld and Hochedlinger, 2010). To avoid this issue, several laboratories have developed integration-dependent gene delivery vectors with incorporated loxP sites that can be subsequently excised from the host genome by transient expression of Cre recombinase (Kaji et al., 2009; Sommer et al., 2009). Transgene-free iPSCs can also be generated with piggyBac transposons, mobile genetic elements that can be introduced and removed from the host genome by transient expression of transposase (Woltjen et al., 2009; Yusa et al., 2009). In order to completely avoid the use of viral particles or plasmid DNA, Warren and colleagues developed a system that achieves efficient conversion of human fibroblasts into iPS cells using direct delivery of RNAs (Warren et al., 2010). Another way to avoid the introduction of exogenous genetic material into donor cells is the deliver of the reprogramming factors as proteins. Specifically,

iPSCs have been derived from both mouse and human fibroblasts by delivering the reprogramming factors as purified recombinant proteins (Zhou et al., 2009) or as whole-cell extracts isolated from either ESCs (Cho et al., 2010) or genetically engineered HEK293 cells (Kim et al., 2009). Although these methods are attractive for the generation of transgene-free iPS cells, the low efficiency of reprogramming and the difficulties in producing the recombinant protein in a reproducible way, make them problematic for their routinely in the laboratory.

## 2.4.2 iPS cells in medicine and biology

The discovery of induced pluripotent stem cells has opened up unprecedented opportunities for the pharmaceutical industry, clinic and research laboratories alike. The fact that self-renewing iPS cells can be derived from any patient offers a unique platform to gain mechanistic insights into a variety of diseases, to carry out *in vitro* drug screening and to explore gene repair strategies coupled with cell-replacement therapies (Fig. 6) (Bellin et al., 2012; Robinton and Daley, 2012; Wu and Hochedlinger, 2011).



**Figure 6. Human iPS cell applications.** iPS cells can be derived from somatic cells of from multiple tissues of patients. After adequate treatments *in vitro*, iPS can be induced to form cells form specialized cells that have several applications, such as disease modeling, drug screening and testing of cellular toxicity response. Adapted from Bellin et al., 2012.

iPS cell technology is especially useful for generating cell lines from patients predisposed to disease, in particular when genetically inherited diseases affect tissues that cannot be easily accessed, such in the case of neuropathologies. Several studies have demonstrated that human iPS cells can be used to model genetic diseases by showing that differentiated cell derivatives affected by the disease in patients recapitulate disease traits *in vitro*. In some situations, patient-specific human iPS cells even seem to be able to reflect the severity of the disease observed in the patient. For example, a progressive loss of motor neurons was observed during *in vitro* differentiation of iPS cells derived from spinal muscular atrophy (SMA) patients, which may reflect the developmental loss of motor neurons seen during this disease (Ebert et al., 2009). Similarly, cardiomyocytes derived from iPS cells from patients with LEOPARD syndrome were found to be enlarged, possibly reflecting the hypertrophic cardiomyopathy associated with this disease (Carvajal-Vergara et al., 2010). Patients suffering from Long QT and Timothy syndrome exhibit increased QT intervals on electrocardiography, and differentiated cardiomyocytes produced from iPSCs from such patients showed prolonged action potentials in single-cell electrophysiological assays (Moretti et al., 2010; Yazawa et al., 2011). Catecholaminergic polymorphic ventricular tachycardia (CPVT) is another congenital disease that is being investigated with the use of human iPS cells. CPVT is a life-threatening disease in young patients who have a structurally normal heart but have an increased susceptibility to arrhythmia under catecholaminergic stress. This feature was clearly evident in human iPS cell-derived cardiomyocytes from patients affected by both the dominant and recessive forms of CPVT compared with healthy controls (Fatima et al., 2011; Jung et al., 2012; Novak et al., 2012). More recently, cardiomyocytes from patient-specific human iPS cells with mutations in SCN5A were shown to exhibit the complex features of a cardiac ‘overlap syndrome’ in which LQTS (Long QT syndrome) coexists with Brugada syndrome (Davis et al., 2012). Many more examples of disease human iPS cell lines exist in the field of neurobiology, perhaps in part due to the availability of more established protocols that allow the generation of differentiated specific neuronal cells. For example, peripheral

neurons from human iPS cells of patients suffering from familial dysautonomia revealed defects in neurogenic differentiation and migration of neuronal precursors due to low expression of IKBKAP (I $\kappa$ B kinase complex associated protein gene), which is involved in transcriptional elongation (Lee et al., 2009). Similarly, alterations in developing neurons from Rett syndrome (RTT) human iPS cells displayed synaptic deficiency, altered Ca<sub>2</sub><sup>+</sup> signaling and electrophysiological defects (Marchetto et al., 2010). Patient-specific human iPS cells that are suitable for modeling Parkinson's disease have also been obtained (Chung et al., 2013; Devine et al., 2011; Ryan et al., 2013). Schizophrenia is another example in which neuronal pathology was demonstrated for a complex genetic psychiatric disorder, and new deregulated pathways that have previously not been associated with schizophrenia were identified (Brennand et al., 2011).

Importantly, in many cases small molecules ameliorating the observed defect in differentiated cells from patient-specific iPSCs have been identified, such as in the case of dysautonomia neuropathy, RETT, LQTS and Parkinson disease (Lee et al., 2009; Marchetto et al., 2010; Moretti et al., 2010; Ryan et al., 2013). Furthermore, human iPS cells from non-monogenetic disorders also provide new clues on possible treatments such as novel antipsychotic drugs for schizophrenia (Brennand et al., 2011) and  $\beta$ -secretase inhibitors for familial and sporadic Alzheimer's disease (Israel et al., 2012). These studies demonstrate the feasibility of using human iPS cell-derived cells for predictive drug screening, and they are a potential starting point to identify effective drug dosages without side effects and to determine how molecules could be modified such as to reduce toxicity while maintaining their therapeutic properties.

Although iPS cells are an invaluable tool for modeling diseases *in vitro*, the goal of developing patient-specific stem cells has also been motivated by the prospect of generating a ready supply of immune-compatible cells and tissues for autologous transplantation. At present, the clinical translation of iPS-cell-based cell therapies seems more futuristic than the *in vitro* use of iPS cells for research and drug development. However, in a proof of principle study, Jaenisch and colleagues used homologous recombination to repair the genetic

defect in iPS cells derived from a humanized mouse model of sickle-cell anemia (Hanna et al., 2007). Directed differentiation of the repaired iPS cells into hematopoietic progenitors followed by transplantation of these cells into the affected mice led to the partial rescue of the disease phenotype. The gene-corrected iPS-cell-derived hematopoietic progenitors showed engraftment in the injected mice and an at least temporal correction of the disease phenotype. Importantly however, a *bona fide* hematopoietic stem cell with the capacity for long-term multilineage reconstitution has yet to be generated from pluripotent cells. In another landmark study from Jaenisch's research group, Wernig and colleagues derived dopaminergic neurons from iPS cells that, when implanted into the brain became functionally integrated and improved the condition of a rat model of Parkinson's disease (Wernig et al., 2008b). The successful implantation and functional recovery in this model is evidence of the therapeutic value of pluripotent stem cells for cell-replacement therapy in the brain, one of the most promising areas for future iPS cell applications.

### **3. MODELS AND EPIGENETICS OF IPS CELL REPROGRAMMING**

Recent progress in high throughput sequencing technologies and the scaling down of the cell numbers required for whole genome analyses has allowed researchers to capture transcriptional and epigenetic snapshots of rare cell populations undergoing cell fate transitions in different biological contexts. These analyses have yielded important insights into the type and sequence of molecular changes inherent to transcription factor induced pluripotency.

#### **3.1 Phases of reprogramming**

The development of improved reprogramming techniques that include homogeneous and inducible reprogramming factor expression systems has enabled a more detailed view of the mechanism underlying reprogramming despite the fact that only a small proportion of somatic cells can be converted in iPSCs. Mouse embryonic fibroblasts (MEFs) are most commonly used as a starting cell type for the dissection of the reprogramming process due to the ease of culture and the possibility of derivation from different genetic backgrounds and mouse models (Papp and Plath, 2013). Current evidence argues that reprogramming to iPS cells requires cell division (Hanna et al., 2009) and that it is a multistep process with two major waves of gene expression changes that coincide with the early extinction of somatic genes and the late activation of core pluripotency genes (Papp and Plath, 2013). Recently, it was shown by the Jaenisch and Hochedlinger groups that the reprogramming process can be dissected based on the expression of cell surface markers (Brambrink et al., 2008; Stadtfeld et al., 2008b). Utilizing specific surface marker combinations, cells in the process of reprogramming were shown to first downregulate the fibroblast-associated marker Thy1 and then upregulate the embryonic marker SSEA1 and finally induce the full pluripotency network (Brambrink et al., 2008; Polo et al., 2012; Stadtfeld et al., 2008a). The downregulation of Thy1 occurs in a large fraction of starting cells, the

subsequent gain of SSEA1 only in a subset of Thy1-negative cells, and the induction of the pluripotency network in a small subset of SSEA1-positive cells, indicating that transitions between each of these steps occur with low probability. Cells that are unable to silence Thy1 become refractory to the action of the reprogramming factors relatively quickly upon OSKM expression and yield iPSCs with a dramatic delay and reduced efficiency (Polo et al., 2012). Genome-wide transcriptional profiling was used to further delineate the sequence of events that drive reprogramming. Initially, cells appear to respond relatively homogeneously to the expression of the reprogramming factors (Polo et al., 2012) and robustly silence typical mesenchymal genes expressed in fibroblasts (such as *Snai1*, *Snai2*, *Zeb1*, and *Zeb2*) (Li et al., 2010; Mikkelsen et al., 2008; Polo et al., 2012; Samavarchi-Tehrani et al., 2010). These events lead to the activation of epithelial markers (such as *Cdh1*, *Epcam*, and *Ocln*) in a process called mesenchymal-to-epithelial transition (MET), which seems to be critical for the early reprogramming phase and is accompanied by morphological changes, increased proliferation, and the formation of cell clusters (Li et al., 2010; Samavarchi-Tehrani et al., 2010). However, the key event is the subsequent gradual activation of pluripotency-associated genes (Golipour et al., 2012; Polo et al., 2012). For example, the pluripotency loci *Nanog* and *Sox2* are transcriptionally upregulated at a late intermediate stage, whereas others, such as *Utf1* or endogenous *Sox2*, are induced even later, closely mirroring the acquisition of the full pluripotency expression program (Buganim et al., 2012). Together, these events culminate in the establishment of the pluripotent state that can be sustained independently of ectopic reprogramming factor expression, as the induced endogenous pluripotency network maintains itself autonomously (Brambrink et al., 2008; Stadtfeld et al., 2008b). Several groups have also reported the transient upregulation of developmental regulators, such as epidermal, extra-embryonic and epiblast-associated genes, at intermediate stages of reprogramming (Hoffmann et al., 2014; Mikkelsen et al., 2008; O'Malley et al., 2013; Polo et al., 2012; Shu et al., 2013). Although the molecular mechanisms underlying this observation remain elusive, it is tempting to speculate that reprogramming intermediates transiently

pass through a state with increased developmental plasticity that could represent stages of normal development (Orkin and Hochedlinger, 2011). Alternatively, these genes might be activated as a consequence of aberrant transcription-factor binding (Sridharan et al., 2009). Regardless, recent studies showed that depletion of some of these transiently expressed genes impairs reprogramming into iPSCs, suggesting functional relevance (Hansson et al., 2012; Hou et al., 2013). Quantitative proteomic analysis during the course of reprogramming of fibroblasts to iPSCs revealed a two-step resetting of the proteome during the first 3 and the last 3 days of reprogramming (Hansson et al., 2012). Proteins related to regulation of gene expression, RNA processing, chromatin organization, mitochondria, metabolism, cell cycle and DNA repair were strongly induced at an early stage, and proteins related to the electron transport system were downregulated. In contrast to these processes, glycolytic enzymes exhibited a slow increase in the intermediate phase, suggesting a gradual transformation of energy metabolism (Zhang et al., 2012). Proteins involved in vesicle-mediated transport, extracellular matrix, cell adhesion and EMT were downregulated in the early phase, retained low levels in intermediate cells and became upregulated in the final stage (Hansson et al., 2012).

These data confirm that reprogramming is a multi-step process characterized by two waves of transcriptome and proteome resetting (Sancho-Martinez and Izpisua Belmonte, 2013). However, because only a small fraction of the induced cells becomes reprogrammed, gene expression profiles of cell populations at different time points after factor induction will not detect changes in rare cells destined to become iPSCs. Therefore a synchronous and high efficiency reprogramming system is needed to address the molecular mechanism of iPS cell reprogramming.

### **3.2 The stochastic and deterministic modes of reprogramming**

As mentioned before, the derivation of iPSCs from most studied somatic cells is extremely inefficient (0.01%–0.1%) and at least 2 weeks (Brambrink et al., 2008; Stadtfeld et al., 2008a). Even in the context of secondary systems, in



which somatic cells homogenously express the factors, the efficiency of fibroblast reprogramming generally does not exceed 1%–5% (Wernig et al., 2008a). Two opposing, but mutually nonexclusive, models have been put forward to explain these observations (Yamanaka, 2009). The so-called “elite” or “deterministic” model proposes that the efficiency of iPSC derivation is low because only a few cells in a somatic cell culture are susceptible to reprogramming. Somatic stem or progenitor cells, present in most adult tissues and possibly also in explanted cell populations, are the most obvious candidates, as they are rare and developmentally closer to pluripotent cells than differentiated cells. In contrast, the “stochastic” model poses that all somatic cells are equally amenable to factor-mediated reprogramming, but have to go through a series of stochastic events to remove epigenetic roadblocks to acquire pluripotency. Only a few cells may overcome all of these roadblocks, resulting in the overall low efficiency. The elite model by itself is difficult to sustain, since iPSC can be derived from several defined somatic cell types, including fully differentiated B and T lymphocytes (Eminli et al., 2009; Hanna et al., 2008) as well as pancreatic  $\beta$  cells (Stadtfield et al., 2008a). Moreover, in clonal populations of early B cells expressing the reprogramming factors almost all cell clones ultimately give rise to daughter cells that form iPSCs, in a process that may require several weeks to months (Hanna et al., 2009). The latter observation suggests that continuous cell proliferation allows rare cells in a homogenous cell population to stochastically acquire changes that facilitate their conversion into a pluripotent state (Hanna et al., 2009).

Recently, Jaenisch and colleagues have employed single cell analyses in an attempt to clarify the different phases of reprogramming. This method allows quantitative analysis of 48 genes in duplicate in 96 single cells and single-molecule mRNA fluorescent *in situ* hybridization to study the different intermediates of the reprogramming process (Buganim et al., 2012). In the first 6 days after factor induction, there was high variation among cells in expression of the 48 genes. This suggests that early in the reprogramming process OSKM factors induce stochastic gene expression changes in a subset of pluripotency genes that is crucial for instigation of the second phase. These stochastic

changes are in addition to the changes in expression of genes that control MET, proliferation and metabolism, which, as mentioned above, occur during reprogramming but are not restricted to cells that are destined to become iPSC (Golipour et al., 2012; Polo et al., 2012). The single-cell analyses also revealed that the stochastic gene expression phase is long and variable (>1 week) and suggested that cells must pass through a rate-limiting stochastic bottleneck before transiting into stable iPSCs (Buganim et al., 2012; Hanna et al., 2009). At a later stage, when the cells start to express *Nanog*, the variation between individual cells in activating pluripotency genes decreases dramatically, which is consistent with a model in which the early 'stochastic' phase of gene expression is followed by a 'deterministic' or more 'hierarchical' phase that leads to activation of the pluripotency circuitry. Although these studies have added knowledge to the field, the development of a robust reprogramming system in which the process is rendered deterministic is essential to study the early molecular events of iPS cells reprogramming.

### **3.3 Epigenetic changes**

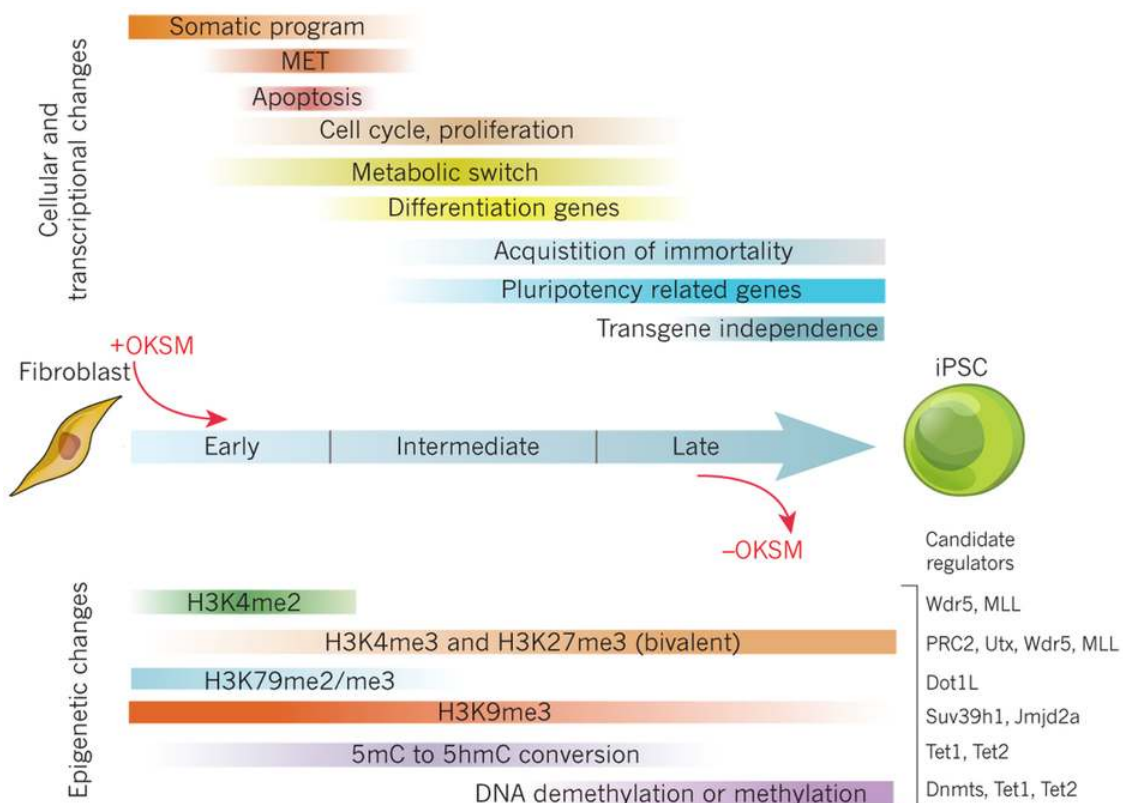
As mentioned before, acquisition of pluripotency is a slow and inefficient process indicating that transcription factors need to overcome a series of epigenetic barriers that are gradually imposed during differentiation to stabilize cell identity and to prevent aberrant cell fate changes. The epigenetic signature of the somatic cell must therefore be erased in order to adopt an ES cell like phenotype and epigenome. These changes include chromatin reorganization, DNA demethylation of promoter regions of pluripotency genes such as *Nanog* and *Oct4*, reactivation of the somatically silenced X-chromosome and genome-wide changes of histone modifications (Fig. 7) (Buganim et al., 2013; Orkin and Hochedlinger, 2011).

#### *Transcription factors drive cell fate change*

*Oct4*, *Sox2* and *Klf4*, three critical components of the pluripotency network, are

the minimal set of factors required for iPSC generation from many cell types under classic reprogramming conditions (Jaenisch and Young, 2008). These factors together suppress lineage-specific genes and activate embryonic stem cell related genes, resulting in the establishment of a self-sustaining pluripotency network (Orkin and Hochedlinger, 2011). By contrast, Myc is unique among the reprogramming factors, as it is neither a component of the core pluripotency network (Kim et al., 2010) nor absolutely necessary for reprogramming to iPSCs (Nakagawa et al., 2008) although it significantly enhances and accelerates reprogramming. Myc expression functions early during reprogramming, presumably by stimulating cell proliferation and inducing a metabolic switch from an oxidative to a glycolytic state that is typical of pluripotent cells (Polo et al., 2012; Sridharan et al., 2009). More recent evidence suggests that Myc contributes to reprogramming by inducing transcriptional amplification of target genes (Lin et al., 2012). Recent studies assessing OSKM occupancy and histone marks early during the reprogramming into iPSCs have provided a first hint on the role of reprogramming transcription factors in dismantling the somatic chromatin and establishing an epigenetic state that is compatible with pluripotency (Koche et al., 2011; Polo et al., 2012; Zhu et al., 2013). Three classes of loci based on chromatin accessibility and timing of transcriptional activation can be categorized from the data collected so far. Genes with an 'open' chromatin state in somatic cells comprise the first group of targets, characterized by increased DNaseI hypersensitivity, active di- and tri- methylation of histone H3 lysine 4 (H3K4me2 and H3K4me3) and the ability to bind OSKM immediately. Downregulated somatic genes and MET genes fall into this group (Polo et al., 2012; Soufi et al., 2012). A second class of early bound OSKM targets includes distal regulatory elements, which seem to require additional chromatin remodeling for transcriptional activation (Soufi et al., 2012). A subgroup of these elements carries the H3K4me1 mark and exhibits nucleosome depletion as well as DNase I hypersensitivity, which are chromatin features characteristic of 'permissive enhancers'. Permissive enhancers typically bind transcription factors before occupancy of their associated promoters and prior to

transcriptional activation (Taberlay et al., 2011).



**Figure 7. Dynamics of key molecular events during direct reprogramming.** A summary of cellular, transcriptional and epigenetic changes (coloured bars) that occur during induced pluripotent stem cell (iPSC) formation from fibroblasts and examples of candidate regulators that have been associated with the depicted chromatin marks in the context of direct reprogramming. Adapted from Apostolou and Hochedlinger, 2013.

The MyoD locus exemplifies this group of enhancers; ectopically expressed Oct4 initially binds to the MyoD enhancer, triggering crosstalk with its promoter and subsequent acquisition of a poised chromatin state (Taberlay et al., 2011). Another subset of distal regulatory elements comprises DNase I resistant loci that are unable to bind Myc alone (Soufi et al., 2012). Early pluripotency genes, such as Sall4, belong to this group. Interestingly, occupancy of these targets by OSK facilitates binding of Myc. This observation thus identifies OSK as “pioneer factors”, as defined by their ability to bind closed somatic chromatin and mediate chromatin remodeling as well as recruitment of other transcription factors and cofactors (Soufi et al., 2012). Broad heterochromatic regions enriched for the repressive H3K9me3 mark constitute a third set of OSKM targets. Genes within this category comprise core pluripotency genes, such as

*Nanog* and *Sox2* (Soufi et al., 2012). These regions are refractory to immediate OSKM binding and seem to require extensive chromatin remodeling for transcriptional activation. These results also suggest that certain histone marks (for example, H3K9 methylation) act as potent barriers that resist acquisition of pluripotency.

#### *Role of histone-modifying enzymes*

Histone marks and chromatin structure are regulated by histone-modifying enzymes such as histone methyltransferases (HMTs), histone acetyltransferases (HATs), histone demethylases (HDMs) and histone deacetylases (HDACs). These enzymes function as co-activators or co-repressors of OSKM at different stages of reprogramming and can influence iPSC derivation (Apostolou and Hochedlinger, 2013). For example, recruitment of Polycomb Repressive Complex 2 (PRC2), which deposits the repressive H3K27me3 mark, and inhibition of Dot1L (which establishes the active H3K79me2 and H3K79me3 marks) have been associated with the downregulation of somatic genes early in reprogramming (Aloia et al., 2013; Fragola et al., 2013; Onder et al., 2012). Accordingly, loss of PRC2 abrogates whereas loss of Dot1L enhances iPSC formation. More in details, loss of DOT1L increases reprogramming efficiency by facilitating loss of H3K79me2 from fibroblast-associated genes, such as the mesenchymal master regulators SNAI1, SNAI2, ZEB1 and TGFB2. Silencing of these genes is essential for proper reprogramming and indirectly increases the expression of the pluripotency genes NANOG and LIN28 (Onder et al., 2012). Activation of the H3K36 histone demethylases Jhdm1a and Jhdm1b (Wang et al., 2011) and suppression of the H3K27 histone demethylase Jmjd3 promote intermediate-to-late stages of iPSC generation by suppressing the expression of the *Ink4/Arf* locus (Zhao et al., 2013), which is essential for the acquisition of immortality. An additional early role for Jhdm1b in epithelial gene activation has recently been reported to mainly regulate epithelial-cell-associated genes and the miR302-367 cluster (Liang et al., 2012). By contrast, H3K9 histone demethylases

maintain the silent heterochromatic state of somatic cells and thus act as major barriers of reprogramming. Consistent with this notion, knockdown of G9a (an H3K9me2 HMT), or Suv39h1 and Suv39h2 and Setdb1 (H3K9me3 HMTs), or overexpression of H3K9 HDMs, increase transcription factor accessibility and result in more efficient iPSC generation from somatic cells (Chen et al., 2013; Soufi et al., 2012; Sridharan et al., 2013). BRG1 (also known as SMARCA4) and BAF155 (also known as SMARCC1), two components of the BAF chromatin-remodeling complex, enhance reprogramming by establishing a euchromatic chromatin state and enhancing binding of reprogramming factors to key reprogramming gene promoters (Singhal et al., 2010). Their overexpression induces OSKM-mediated demethylation of pluripotency genes such as *Oct4*, *Nanog* and *Rex1* (also known as *Zfp42*) and enhances conversion to iPSCs (Singhal et al., 2010). Reprogramming transcription factors have been reported to directly interact with histone-modifying enzymes, providing a mechanistic explanation for how they may alter chromatin and cell state during induced pluripotency. Examples include Utx and Wdr5 that bind to Oct4 protein and co-occupy many genomic targets in ES cells. Depletion of either factor impairs iPSC formation (Ang et al., 2011; Mansour et al., 2012). Intriguingly, some reprogramming-associated cofactors function in a chromatin-independent manner. For example, Jmjd3 blocks reprogramming not only by activating the *Ink4a/Arf* locus but also by targeting the methyl-lysine effector protein Phf20 for ubiquitination; which is required to activate Oct4 transcription in collaboration with Wdr5 (Zhao et al., 2013).

### *DNA methylation*

DNA methylation is considered to be the most stable epigenetic modification, which confers permanent gene silencing both during development and in the adult. Changes in histone modifications typically precede the removal or the deposition of DNA methylation marks during differentiation (Gifford et al., 2013). Similarly, DNA methylation changes at pluripotency loci almost exclusively occur at the end of the reprogramming process (Polo et al., 2012). DNA

methylation is established by the *de novo* methyltransferases Dnmt3a and Dnmt3b and preserved by the maintenance methyltransferase Dnmt1. Deletion of the mouse enzymes Dnmt3a and Dnmt3b has no consequence for cellular reprogramming (Pawlak and Jaenisch, 2011). In contrast to the dispensability of *de novo* methylation for iPSC formation, DNA demethylation of pluripotency genes seems to be crucial. Demethylation can occur by either active or passive mechanisms (Kohli and Zhang, 2013) both of which have been implicated in iPSC generation. Tet enzymes associated with active DNA demethylation have recently been described to be implicated in iPS reprogramming. TET proteins catalyze the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which serves as a substrate for thymine DNA glycosylase (TDG)-mediated base excision repair into unmodified cytosine (Kohli and Zhang, 2013). After overexpression of OSKM, Tet2 induces hydroxymethylation of key pluripotency genes such as Nanog and Esrrb, priming them for subsequent demethylation and transcriptional activation (Doege et al., 2012). Interestingly, proteomic and genomic analyses revealed that Tet1 and Tet2 directly interact with Nanog and co-occupy many pluripotency targets in ES cells, implicating Nanog in the targeting of Tets (Costa et al., 2013). In agreement, simultaneous overexpression of Tet1 or Tet2 together with Nanog significantly enhances iPSC formation (Costa et al., 2013; Doege et al., 2012). Moreover, Tet1 overexpression can compensate for exogenous Oct4 expression during cellular reprogramming (Gao et al., 2013). Inefficient DNA demethylation or remethylation has further been suggested to be the main reason for the 'epigenetic memory' observed in many iPSC lines (Robinton and Daley, 2012).

### *Three-dimensional chromatin architecture in reprogramming*

Accumulating evidence suggests that local and three-dimensional (3D) chromatin architecture provide additional levels of gene regulation in pluripotent stem cells (de Laat and Duboule, 2013). However, their roles in cellular reprogramming are only partly understood. Histone variants usually modify the

ability of nucleosomes to undergo remodeling (Apostolou and Hochedlinger, 2013). In particular, the histone variant macroH2A has previously been associated with resistance to efficient chromatin remodeling (Skene and Henikoff, 2013). In agreement, the presence of macroH2A potentially inhibits transcription-factor-induced reprogramming of somatic cells to pluripotency by maintaining pluripotency loci in a repressed state (Barrero et al., 2013; Gaspar-Maia et al., 2013; Pasque et al., 2012). In addition to local chromatin structure, 3D chromatin architecture has been implicated in pluripotency, differentiation and reprogramming (Apostolou and Hochedlinger, 2013; de Laat and Duboule, 2013). Differentiation of ES cells is accompanied by repositioning of pluripotency genes from the nuclear center to the nuclear periphery (Peric-Hupkes et al., 2010) and a disruption of promoter-enhancer looping at key pluripotency loci such as *Oct4* and *Nanog* (Gaspar-Maia et al., 2013; Kagey et al., 2010; Phillips-Cremins et al., 2013). A recent study identified complex pluripotency specific long-range interactions of the *Nanog* locus, which become rearranged during differentiation and are reestablished during reprogramming (Apostolou et al., 2013). Extending these findings, long-range chromatin interactions involving the *Oct4* promoter region were recently implicated in the reprogramming of murine and human cells (Wei et al., 2013; Zhang et al., 2013). Importantly, these interactions took place specifically in those rare cells that were poised to form iPSCs, and they preceded transcriptional activation, suggesting a causal effect for 3D chromatin structure on transcription (Wei et al., 2013; Zhang et al., 2013).



## **4. C/EBP $\alpha$ and cell reprogramming**

A study from Jaenisch and colleagues in 2008 has shown for the first time the possibility to reprogram terminally differentiated cells to pluripotency by defined factors (Hanna et al., 2008). Initially, they observed that the OSKM factors were not sufficient to induce reprogramming in terminally differentiated B cells and that an additional factor was required to destabilize the transcriptional state maintaining the B cell state. This was obtained either by overexpressing the transcription factor C/EBP $\alpha$ , or by knockdown of Pax5, a key transcription factor in B cells. Further work demonstrated the ability of C/EBP $\alpha$  to enhance about 10 times the number of iPS cell colonies obtained from pre-B cells, reaching an efficiency of 2-3% (Eminli et al., 2009).

### **4.1 CCAAT/enhancer-binding protein alpha**

The CCAAT/enhancer-binding protein (C/EBP) family comprises six members. Each member is designated by a Greek letter, corresponding to the chronological order of their discovery: C/EBP  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$  (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 leucine zipper domain, C/EBP proteins can form homo- and heterodimers in all intrafamilial combinations (Ramji and Foka, 2002).

C/EBP $\alpha$  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/EBP $\alpha$  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 recruits chromatin remodeling complexes (SWI/SNF) (Koschmieder et al., 2009). The capacity of C/EBP $\alpha$  to promote differentiation in a tissue specific manner is thought to rest on its ability to

collaborate with other transcription factors such as PPAR $\gamma$  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/EBP $\alpha$  in tissue development has emerged from the study of C/EBP $\alpha$  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/EBP $\alpha$   $-/-$  mice reveal that the lack of mature granulocytes is due to the lack of granulocyte-monocyte progenitors (GMPs), while the number of common myeloid progenitors (CMPs) is normal (Zhang et al., 2004). In fact, conditional deletion of C/EBP $\alpha$  in GMPs allows for normal granulopoiesis *in vitro*, indicating that C/EBP $\alpha$  is not required in granulopoietic differentiation beyond the GMP stage (Zhang et al., 2004). C/EBP $\alpha$  also controls fetal liver HSC self-renewal as C/EBP $\alpha$   $-/-$  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/EBP $\alpha$  is essential for the formation and maintenance of adult HSCs (Ye et al., 2013).

C/EBP $\alpha$  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/EBP $\alpha$  is an intronless gene it encodes two different proteins isoforms generated by regulated alternative translation initiation of the same mRNA: a full length protein (p42) and a truncated one (p30) that differ in their N-terminus. The C/EBP $\alpha$  long isoform p42 is able to promote cell cycle arrest through different mechanisms, including interaction with Cdk2, Cdk4 and SWI/SNF chromatin complex, upregulation of p21 and repression of E2F that leads to myc downregulation (Johnson, 2005; Zhang et al., 2004). Interestingly the short isoform p30 lacking the N-terminus lacks antimitotic activity but is able to promote cell differentiation (Calkhoven et al., 2000). For instance, it has been shown that mice lacking p42 but retaining the p30 isoform have GMPs and myeloid progenitors but fail to fully differentiate and when these cells are placed in culture they hyperproliferate (Kirstetter et al., 2008).

## 4.2 Transdifferentiation of murine B cells into macrophages

Previously, our group reported that forced expression of C/EBP $\alpha$  could rapidly and efficiently convert mouse primary B cells them into macrophages (Xie et al., 2004). Transdifferentiation has been shown to occur also *in vivo*. Using B cell precursors (B220<sup>+</sup>) from lineage tracing mice (CD19Rosa26 EYFP), in which B cells are permanently labeled in green, were purified and infected with C/EBP $\alpha$  hCD4 retrovirus and injected into sublethally irradiated Rag2<sup>-/-</sup> gc <sup>-/-</sup> mice. 6 days after injection 51% of the C/EBP $\alpha$  EYFP<sup>+</sup>hCD4<sup>+</sup> cells were CD19-Mac-1<sup>+</sup> in the bone marrow and 32% in the spleen, precisely reflecting the timing and percentages observed *in vitro*.

Since the cell number of primary B cell precursors is limited and their culture require stromal cells, making quite challenging the study of the molecular events underlying the transdifferentiation process. Therefore, a robust transdifferentiation system was developed, consisting of a clone of a pre-B cell line (HAFTL) expressing an inducible form of C/EBP $\alpha$  fused to the estrogen receptor (ER) and GFP. The C/EBP $\alpha$ ER-GFP expressing cells can be converted by  $\beta$ -estradiol, in the absence of stromal cells, into macrophages like cells at 100% efficiency in 2 to 3 days (Bussmann et al., 2009). The obtained macrophages cells are large, highly migratory and phagocytic and exhibit an inflammatory response to LPS. After induction of C/EBP $\alpha$  several thousand genes (including *lyzs*, *csf1r*, *fcgr1*) become upregulated and several thousand genes (including B cell genes such as *vpreb1*, *pax5*, *ebf1*) become downregulated. In addition, 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 day (Bussmann et al., 2009).

The use of this robust and efficient transdifferentiation system allowed to study the question if during transdifferentiation the cells pass trough and intermediate progenitor stage or if they even retrodifferentiate to a pluripotent state before becoming macrophages. Studies performed in our laboratory 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/EBP $\alpha$  was activated in pre-B cells under culture conditions permissive for HSCP cell growth or when C/EBP $\alpha$  was activated in a time limited fashion (Di Tullio et al., 2011). Taken together these data showed that the B to macrophage switch is a direct process that does not involve retrodifferentiation. 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/EBP $\alpha$  approximately 90% of the cells divide once or twice, while 8% do not divide at all before acquiring a macrophage phenotype. Importantly, the non dividing subset express the highest level of C/EBP $\alpha$  and is the fastest in differentiating, suggesting that high levels of C/EBP $\alpha$  accelerate both the switching process as well as cell cycle arrest. (Di Tullio and Graf, 2012a). 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, H3K14ac and H3K4me3) and repressive (H3K27me3) histone modification marks changed according to the expression levels of these genes (Rodriguez-Ubreva et al., 2012). However, more detailed studies using both the inducible pre-B cell line and primary B cells have shown a role for the enzyme Tet2 in transdifferentiation. C/EBP $\alpha$  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 have 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 the MEF2c transcription factor to silence myeloid genes. During the conversion from pre-B cells to macrophages HDAC7 expression is downregulated, relieving the repression. 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).

Recently our group has been studying the molecular mechanism by which C/EBP $\alpha$  induces transdifferentiation, by activating a new gene expression program while silencing the old one. In details, genome-wide ChIP-seq binding profiles of C/EBP $\alpha$ , C/EBP $\beta$  and PU.1 and the lymphoid factors E2A, Ebf1, Pax5 and Foxo1 were generated using the pre B cells line containing C/EBP $\alpha$ ER line. These data were analyzed in combination with ChIPseq experiments for the enhancer chromatin marks H3K4me1 and H3K27Ac, the promoter marks H3K4me3 and H3K27me3 and for the binding of the co-activator p300 and polymerase II at different times during the conversion of B cells to macrophages, and correlated with changes in expression of adjacent genes. Two distinct types of macrophage enhancer were identified used by C/EBP $\alpha$  to build up the myeloid program. For instance, C/EBP $\alpha$  acts either as a pioneer factor by binding sites within closed chromatin configuration or as a secondary factor, by binding to myeloid enhancers already primed by PU.1 binding. In contrast, to silence the B cell program C/EBP $\alpha$  was found to transiently bind to 'super-enhancers' of the B cell master regulators Ebf1, Pax5 and Foxo1. This leads to the local release of p300, deacetylation of H3K27 and stalling of polymerase II, resulting in the inactivation of these enhancers and the rapid downregulation of the associated factor-encoding genes. As a consequence, the autoregulatory loops of these master regulators become interrupted and the whole B cell program collapses. In conclusion, these data show that C/EBP $\alpha$  induces the complete conversion of B cells into macrophages by orchestrating the activation and inactivation of enhancers associated with myeloid and lymphoid gene

**Aims**

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Conversion of somatic cells to pluripotency by defined transcription factors is a long, inefficient and complex process. In order to improve the quality of resulting induced pluripotent stem cells, of extreme relevance for potential therapeutic applications, and to address fundamental questions about control of cell identity, molecular mechanisms of the reprogramming process must be understood.

The main aim of my work was to test whether a system can be developed with which iPS reprogramming can be achieved rapidly and at high efficiencies, suitable to discover the mechanism controlling the process of somatic cell reprogramming to pluripotency.

## **PART II**

### **RESULTS**



## **C/EBP $\alpha$ poises B cells for rapid reprogramming into iPS cells**

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**PART III**  
**DISCUSSION AND CONCLUSIONS**

## **Discussion**

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Resetting the epigenome of a somatic cell to a pluripotent state has been achieved by somatic cell nuclear transfer (SCNT), cell fusion and ectopic expression of defined factors (Jaenisch and Young, 2008). Understanding the molecular mechanisms that underlie somatic cell reprogramming to pluripotency is crucial for the creation of high-quality pluripotent cells and may be useful for therapeutic applications. Moreover, insights gained from *in vitro* reprogramming approaches may broaden our understanding of fundamental questions regarding cell plasticity, cell identity and cell fate decisions. Unfortunately, iPSC cell reprogramming is lengthy, inefficient and generates induced pluripotent stem cells (iPSCs) that vary widely in their developmental potential. Developing a deterministic reprogramming system by removing the barriers that resist the induction of pluripotency would represent an invaluable tool to dissect mechanisms underlying stem cell self-renewal and cellular differentiation. Previous work by Hanna and colleagues documented that clonal somatic cell populations invariably produced iPSCs, albeit at different latencies, when given additional time to divide in culture (up to 18 weeks) (Hanna et al., 2009). These observations raised the fundamental question of whether factors could be identified whose manipulation would convert iPSC induction into a deterministic process.

We have now shown that an 18h pulse of C/EBP $\alpha$  followed by expression of the OSKM pluripotency factors reprograms B cells to iPSCs at a 100-fold higher efficiency than unsensitized controls. Essentially all known core pluripotency genes become upregulated, reaching levels comparable to ESCs within one week. This occurs in two waves, with *Oct4*, *Lin28*, *Tdh*, *Gdf3* and *Zfp296* being upregulated within 2 days followed by *Nanog*, *Sox2*, *Esrrb*, *Sall4*, *Zfp42* and *Dppa5a*. In addition, genes known to be expressed after at least 3 weeks during reprogramming to pluripotency from MEFs (Golipour et al., 2012) are immediately activated in our system. Equally compressed in time is the early deregulation of the mesenchymal genes *Snail*, *Slug* and *Tgfb2* and of the epithelial genes *Cdh1* and claudins that participate in the mesenchymal-epithelial transition. This suggests that MET and pluripotency network activation

occur concomitantly, unlike in other systems described (Li et al., 2010; Samavarchi-Tehrani et al., 2010). Moreover, C/EBP $\alpha$  directly bind to TGF $\beta$  pathway genes, regulating their expression and favoring the initiation in B cells of an EMT, reminiscent of the recently described EMT-MET during MEF reprogramming (Liu et al., 2013).

The downregulation of the somatic program is an essential step that takes place before the pluripotency network is established (Apostolou and Hochedlinger, 2013). In fact, in our system, the cells respond homogenously at the C/EBP $\alpha$  pulse with the majority of known B cell factors being rapidly downregulated.

Single cell cloning experiments, gene expression changes and proportion of Oct4-GFP positive cells suggest that 95% of the cells become reprogrammed within 1 week. Under serum-free conditions the C/EBP $\alpha$  effect is even more dramatic, poising >60% of the cells to become Oct4-GFP in 2 days. A similar high reprogramming efficiency has recently been reported for cells with an ablation of Mbd3 (Rais et al., 2013), but unlike cells reprogrammed with this deficiency  $\alpha$ iPSCs are not modified genetically. Strikingly myeloid progenitors, whose formation requires C/EBP $\alpha$  (Zhang et al., 1997), exhibited the highest reprogramming efficiency among somatic cell types tested so far (25%) (Eminli et al., 2009).

What is the mechanism of the C/EBP $\alpha$  effect? In addition to the already discussed ability of CEBP $\alpha$  to initiate both an EMT transition and to collapse of the B cells program, we have reported complementary molecular mechanisms linked to the CEBP $\alpha$  pulse. Indeed, a striking finding was that the *Oct4* promoter became partially demethylated 2 days after OSKM induction, paralleled by the gene's accessibility to *Oct4* binding and its transcriptional activation. Several lines of evidence suggest that this process involves the activity of the Tet2 dioxygenase since Tet2 overexpression enhances B cell reprogramming, the C/EBP $\alpha$  pulse upregulates *Tet2* by direct binding to upstream sites and also shuttles the protein into the nucleus in what appears to be a novel mechanism of Tet2 activation. Here Tet2 binds to pluripotency genes where it converts 5mC

residues into 5hmC. This modification could either directly cause the gene's de-repression as observed during B cell to macrophage transdifferentiation (Kallin et al., 2012) or after subsequent DNA demethylation or both (Bagci and Fisher, 2013).

Why is the C/EBP $\alpha$  pulse more powerful than its co-expression with OSKM? Since it has been described that the 5hmC mark is sufficient to induce chromatin remodeling (Doege et al., 2012; Yildirim et al., 2011), we perform DNase I sensitivity assay at pluripotency genes bound by Tet2. In fact, we observed increased chromatin accessibility at all sites tested in 18h-pulsed cells, whereas the same loci and control regions showed no chromatin remodeling. Importantly, when C/EBP $\alpha$  expression was sustained for more than 18h the chromatin at pluripotency genes returned to his original closed state. In conclusion, C/EBP $\alpha$  transiently opens large stretches of chromatin through epigenetic modifications that become re-set when the cells reach their new end stage.

The fact that sustained C/EBP $\alpha$  overexpression induces a B to macrophage transdifferentiation raised the possibility that its capacity to sensitize B cells to iPSC reprogramming is a general feature of lineage-instructive transcription factors. However, the effect of C/EBP $\alpha$  is highly specific, as only the closely related factor C/EBP $\beta$  but neither GATA-1, Mash1 nor MyoD mediate enhanced iPSC reprogramming. The C/EBP $\alpha$  effect might recapitulate the gene's function in early embryo development since it is co-expressed with pluripotency factors in mouse morulae and sequestered within the trophectoderm at the blastocyst stage (Guo et al., 2010). Subsequently it is required, in combination with C/EBP $\beta$ , for the formation of trophoblasts in the placenta, cells that share functional and gene expression properties with macrophages (Guilbert et al., 1993; Holland et al., 2004).

Finally, it will be interesting to determine how C/EBP $\alpha$  mediates nuclear translocation of Tet2 and how the enzyme is recruited to its target genes.

The system described should help to define the precise sequence of transcriptional and epigenetic events leading to completely reprogrammed iPSCs. And the path-breaker effect of C/EBP $\alpha$ , if reproducible in human cells, might yield high quality pluripotent cells (Okada and Yoneda, 2011) potentially useful for regenerative medicine.

## **Conclusions**

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1. An 18 hour pulse of C/EBP $\alpha$  expression followed by OSKM activation induces a dramatic increase in the iPSC reprogramming efficiency, involving up to 95% of the cells within 4 days;
2. Oct4-GFP positive cell appear at day 2;
3. Pluripotency and epithelial-mesenchymal transition genes become concomitantly upregulated, reaching levels similar to those seen in embryonic stem and iPS cells within 1 week;
4.  $\alpha$ iPSCs are pluripotent and have full developmental potential both *in vivo* and *in vitro*;
5. Tet2 is partially responsible for the increased efficiency in iPS reprogramming. ChIPseq experiments show that C/EBP $\alpha$  binds to the *Tet2* gene, RNAseq that it induces its expression and immunofluorescence that it translocates Tet2 to the nucleus;
6. Co-expression of Tet2 with OSKM enhances iPS reprogramming efficiency significantly;
7. C/EBP $\alpha$  acts as a path-breaker. For instance, the pulse renders the chromatin at regulatory sites of pluripotency genes accessible to DNase I and, following OSKM induction, these sites become demethylated and accessible to the binding of Oct4.
8. The effect of C/EBP $\alpha$  is both transcription factor- and cell type specific

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## ANNEX I

### PUBLICATIONS

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## ANNEX II

### ABBREVIATIONS

OSKM	Oct4, Sox2, Klf4 and c-Myc
Yamanaka factors	Oct4, Sox2, Klf4 and c-Myc
iPSCs	induced pluripotent stem cells
ICM	inner cell mass
ESCs	embryonic stem cells
EE	extra-embryonic
ECCs	embryonic carcinoma cells
TSC	trophoblast stem cell
PGCs	primordial germ cells
EGCs	embryonic germ cells
GSCs	germline stem cells
EpiSCs	epiblast-derived stem cells
LIF	leukemia inhibitory factor
BMP4	bone morphogenetic protein 4
MAPK	mitogen-activated protein kinase
GSK3	glycogen synthase kinase-3
SCNT	somatic cell nuclear transfer
STAP	stimulus-triggered acquisition of pluripotency
TERT	telomerase reverse transcriptase
miRNAs	MicroRNAs
MET	mesenchymal to epithelial transition
MECP2	DNA-binding protein genes methyl-CpG binding protein 2
MBD2	methyl-CpG binding domain protein 2
ALKi	anaplastic lymphoma kinase inhibitor
VPA	valproic acid
MMLV	moloney murine leukaemia virus
rtta	tetracycline-controlled transactivator
SMA	spinal muscular atrophy
CPVT	Catecholaminergic polymorphic ventricular tachycardia
IKBKAP	I $\kappa$ B kinase complex associated protein gene
RTT	Rett syndrome
MEFs	Mouse embryonic fibroblasts
HMTs	histone methyltransferases
HATs	histone acetyltransferases
HDMs	histone demethylases
HDACs	histone deacetylases
PRC2	Polycomb Repressive Complex 2
5-mC	5-methylcytosine
5-hmC	5-hydroxymethylcytosine
TDG	thymine DNA glycosylase
3D	three-dimensional

C/EBP	CCAAT/enhancer-binding protein
b-ZIP domain	leucine zipper dimerization motif
GMPs	granulocyte-monocyte progenitors
CMFs	common myeloid progenitors
HSC	Hematopoietic stem cells
ER	estrogen receptor
HAFTL	pre-B cell line
HSCP	HSCs and progenitors
E2	$\beta$ -estradiol
B+OSKM cells	B cells induced with OSKM
B $\alpha$ ' + OSKM cells	B cells induced for 18h with C/EBP $\alpha$ and then induced with OSKM
EMT	epithelial to mesenchymal transition
GSEA	Gene Set Enrichment Analysis
AP	Alkaline Phosphatase
EBs	Embryoid bodies
RIN	RNA integrity number
ChIP	Chromatin immunoprecipitation
hMeDIP	5-hydroxymethylcytosine (5-hmC) methylated DNA immunoprecipitation