Reprogramming hierarchy in mouse liver cell subpopulations depends on cell intrinsic factors including BAF complex members.

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By

Pallavi Mahaddalkar, M.Res. Medical and Molecular Biosciences

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Institute of Molecular Medicine and

Max-Planck Research Group on Stem Cell Aging

Universität Ulm, Germany

Faculty of Natural sciences

Dekan: Prof. Dr. Joachim Ankerhold

Prüfungsvorsitzender : Prof. Dr. Axel Brennicke

1. Gutachter : Prof. Dr. med. Karl Lenhard Rudolph

2. Gutachter : Prof. Dr. med. Christian Buske

1. Wahlmitglied : PD Dr. Stefan Liebau

2. Wahlmitglied : Prof. Dr. Niel Johnsson

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1 Declaration

I hereby declare that I wrote the present dissertation with the topic: **Reprogramming hierarchy in mouse liver cell subpopulations depends on cell intrinsic factors including BAF complex members** independently and used no other aids that those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current „Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis“

Ulm, May 20, 2013

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Pallavi Mahaddalkar

2 Acknowledgement

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4 Abstract

Ectopic expression of certain transcription factors induces reprogramming of somatic cells to a pluripotent state. Accumulating evidence in the field of induced pluripotent stem cells (iPSCs) points towards an increasing need of fast and efficient reprogramming strategies to generate patient specific iPS cells. However, the limited knowledge regarding the molecular mechanisms that can possibly govern the process of reprogramming still poses as a challenge to overcome the low reprogramming efficiencies. Recently, various studies have tried to address this impounding question by reprogramming various cell populations. It had been successfully shown that stem/progenitor cells derived from different organs reprogram more efficiently than their differentiated counterparts thus unveiling a hierarchical reprogramming efficiency in the cells from different organs. Nevertheless, the molecular mechanisms governing this graded reprogramming phenomenon were yet to be explored. To analyse the molecular players behind this grading in reprogramming, we aimed to investigate the reprogramming capacity of different types of cells from liver parenchyma, an endodermal derived organ.

In our study we found that different types of liver cells exhibit a kind of hierarchy during reprogramming towards induced pluripotent stem cells. More precisely, liver progenitor cells (LPCs) showed 275-fold superiority in reprogramming compared to differentiated liver cells (non-LPCs). When subjected to various molecular tests, LPCs endogenously express certain reprogramming factors but omission of those factors still did not allow successful reprogramming. On the other hand, LPCs showed higher reprogramming efficiencies with similar proliferation rates as their differentiated equivalents thus ruling out the effect of proliferation but impressing on the association of expression of endogenous pluripotency factors on higher reprogramming efficiencies. The presence of BAF (Brg1/Brm associated factor)-complex members Baf155 and Brg1 appeared to mediate the superior reprogramming in LPCs compared to non-LPCs as the knockdown of these BAF complex members annulled the increase reprogramming efficiencies of LPCs compared to non-LPCs[1].

Together our results suggest a possible mechanism underlying higher reprogramming in stem/progenitor cells compared to their differentiated counterparts. The study also put forth a possible cell source for fast and efficient reprogramming.

5 Introduction

5.1 Preimplantation development and stem cell classes

Mammalian embryogenesis is the process of fertilisation of a human oocyte by a sperm to form a diploid zygote, resulting in implantation and further development and differentiation. After fertilisation, cells undergo a consecutive cleavage and finally at 3d p.c. a fully developed blastocyst is formed. A fully developed blastocyst comprises of an embryoblast or so-called inner cell mass (ICM), a trophoblast and the blastocoel. Both the trophoblast and the inner cell mass have different cell fates during embryonic development [2]. Trophoblast cells have a major role during implantation of the embryo in the uterus and later in the nourishment of the embryo by forming extra embryonic tissues like the placenta [3]. The inner cell mass (ICM) is characterised by 50-150 cells depending on the days after fertilisation. Those cells are located in the blastocyst cavity and later on will form the epiblast of the pre-gastrula embryo [3]. The cells of the ICM are the cells which differentiate into the 3 germ layers ectoderm, endoderm and mesoderm (**Figure 1**). Mammalian development starts with a diploid zygote and concludes in establishment of various types of specialised cells. As the cells specialise, the cells are restricted in their developmental potential. The potential of these cells to differentiate to various other cell types terms the potency of the cells. Stem cells are classified on the basis of their cell potency and plasticity as totipotent cells, pluripotent cells, multipotent cells and unipotent cells (**Figure 2**) [3].

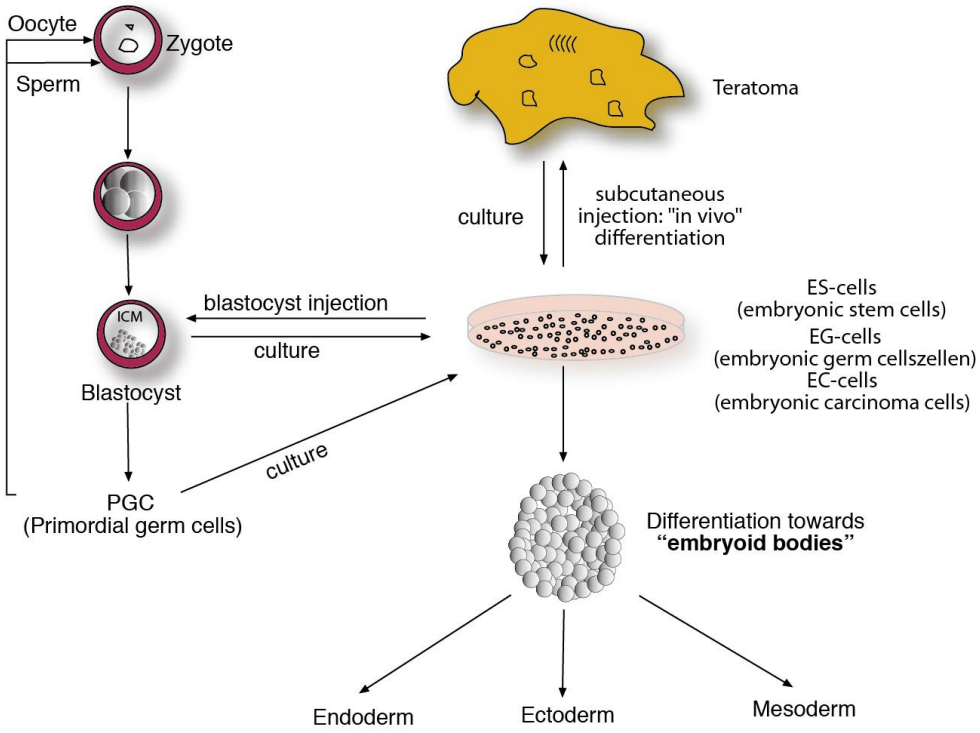


Figure 1 Origin and determination of pluripotent cells. After fertilisation of the oocyte by the sperm, the cells of the zygote multiply to form a blastocysts containing inner cell mass (ICM). *In vitro*, the cells from the blastocysts can be cultured to give rise to different cell lines. These cells also maintain their potential to differentiate *in vitro* into all three germ layers and can give rise to teratomas *in vivo* when injected into NOD-SCID mice.([4] Adapted from Dr. A. Kleger).

Totipotent cells have the ability to produce fully differentiated cells including the extra embryonic tissues. Totipotent cells can give rise to an entire organism. In mammals, only the zygote and the cells of the first cleavage blastomeres contribute have a totipotent capacity [5].

Pluripotent cells have the ability to differentiate into all three germ layers but lack the ability to contribute to the extra embryonic lineage such as the placenta. This lack of ability to form extra embryonic tissues restricts these cells from forming a whole

organism. Inner cell mass, epiblast, primordial germ cells and gametes are considered as *in vivo* examples of pluripotent stem cells whereas ESCs, epiblast stem cells, embryonic germ cells and spermatogonial stem cells are the in vitro pluripotent counterparts [6, 7]. Recently, differentiated stem cells are induced with over expression vector cocktail of pluripotent genes such as Oct3/4, Sox2, cMyc and Klf4 for generation of pluripotent cells in vitro. These cells are defined to be induced pluripotent stem cells or iPSCs [5].

Multipotent cells are cells which can self-renew and but have a more restricted differentiation potential. These cells are also called adult stem cells as they remain in adult tissues [8]. These cells can differentiate into different cells from specific lineage. Unlike pluripotent stem cells, multipotent stem cells serve to regenerate and/or repair the respective organ of the body throughout the lifetime of an organism by replenishing the body with differentiated cells after an injury like haematopoietic stem cells (HSCs) [9] or intestinal stem cells (ISCs) [10].

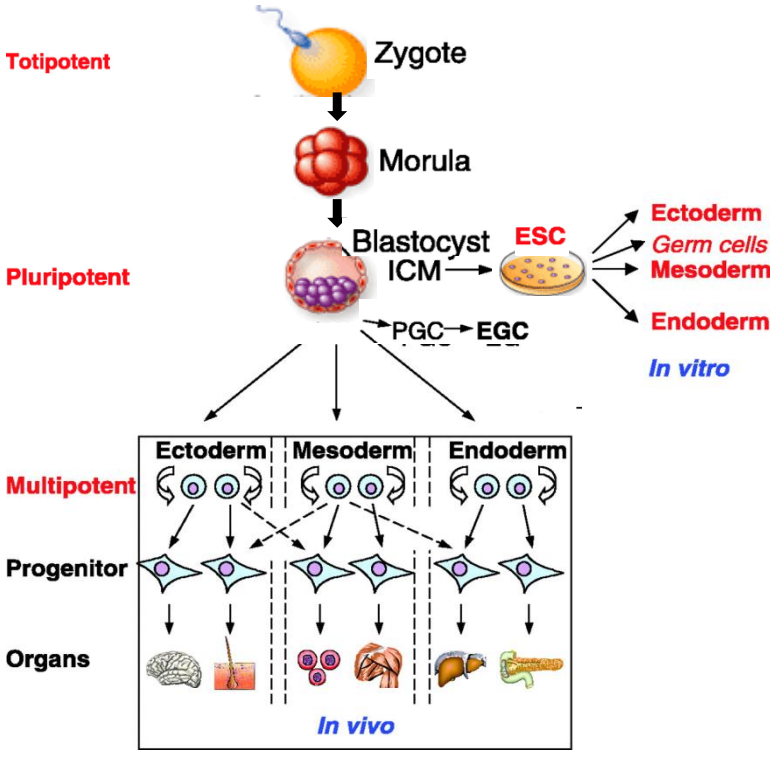


Figure 2 Hierarchy of cellular potency during mammalian development. During mammalian development, the differentiation potential of a cell becomes restricted as it becomes more specialized and more committed to the respective lineage [4].

5.2 Regulation of pluripotency

5.2.1 Signalling cascades

In 1981, cells of the mouse ICM were first isolated and subjected to cultural conditions similar to that of embryonic carcinoma (EC) cells. These cells were termed as the Embryonic stem cells (ESCs) [11, 12]. ESCs are characterised by their main characteristics of self-renewal, pluripotency, teratoma formation and germ line contribution upon blastocysts injection and subsequent transfer into pseudo-pregnant mice. Self-renewal is the property of ESCs by which they can undergo mitotic cell divisions and expand indefinitely while maintaining their pluripotent state [13]. Several years later the human cells from ICM were isolated in vitro and human ES cell lines were established **(Figure 1)** [14].

Pluripotent cells gradually form teratomas upon injection into immune-deficient mice. Those ESC derived teratomas show all differentiated cell types from the three germ layers. The formation of teratomas is also considered to be a stringent *in vivo* test used to characterise pluripotent cells **(Figure 1)**. The most rigid test for pluripotency in the mouse is the formation of chimera and germ line contribution of the ESCs. The donor ESCs when injected in the diploid blastocysts are known to incorporate into the host ICM. When these blastocysts are implanted into a surrogate mother a chimera can be generated (a chimera mouse consisting of host ICM- and ESC derived tissues from all germ layers) [15, 16]. The most rigorous test for pluripotency is tetraploid embryo complementation, where ESCs are injected into a tetraploid (4n) host blastocyst, generated by fusing a diploid 2-cell embryo. Tetraploid cells cannot contribute to somatic lineages of the embryo and therefore the embryo is exclusively derived from the injected ESCs [17, 18].

Pluripotency is governed by a complex but strict array of molecular mechanisms as well as core pluripotency factors which maintain either the naïve state or the primed state of the ESCs. The naïve state of pluripotency is characterized by small domed shaped colonies; high clonal expansion capacity from single cells and is tightly controlled by the LIF/Stat3, BMP4 and Wnt mechanisms [7, 19]. On the other hand, the primed pluripotent state is characterized by low clonal expansion of cells from singe cells, more flattened colony morphology and the involvement of TGFß and FGF pathways which induced differentiation in the naïve pluripotent state [7, 19].

Initially, ESCs were isolated and plated on inactivated mouse embryonic fibroblasts (MEFs). It was later found that the MEFs secrete the cytokine LIF that activates the JAK pathway by heterodimerization of LIF receptors with gp130. The activated JAK pathway leads to phosphorylation of tyrosine receptors which dock the Stat family transcription factors mainly STAT-3. These phosphorylated STAT-3 protein in turn mediates the expression of genes which are essential for self renewal in ESCs [20-22]. Serum factors like bone morphogenic protein 4 (BMP4) have been identified to have a dual role in controlling self-renewal as well as differentiation of the ESCs. On the one hand, the activation of BMP4 leads to the inhibition of differentiation genes by phosphorylating the Smad family transcription factors thus promoting self-renewal of the ESCs while on the other hand, in absence of LIF, the presence of BMP4 drives the differentiation of ESCs towards mesodermal lineage suggesting a prominent role of these pathways in ESC self-renewal [23-25]. Thus, BMP4 acts in concert with LIF/Stat3 signaling pathway to inhibit lineage specific genes and, thus, maintain self-renewal in ESCs.

Another important signalling cascades in ESCs is the fibroblast growth factor 4 (FGF-4), glycogen synthase kinase 3 ß (GSK3ß) pathway and mitogen activated protein kinase (MAPK) pathway. FGF-4-FGFR pathway and MAPK pathway regulates fate decisions of ESCs via ERK1/2 pathway [26] while the activation of GSK3ß induce the differentiation process via inhibiting Wnt signalling [27] . It has been already reported that the inhibition of ERK1/2 pathway improves stemness of ESCs by enhancing the self-renewal of the ESCs **(Figure 3)** [27-32]. In an approach proposed by the Smith

lab, a screen of small compounds revealed inhibitors, which could successfully inhibit the two protein kinases namely GSK3ß and ERK1/2. In presence of LIF both the inhibitors successfully maintained pluripotency of the ESCs without the need of feeder cells or serum **(Figure 3)** [27, 30-32].

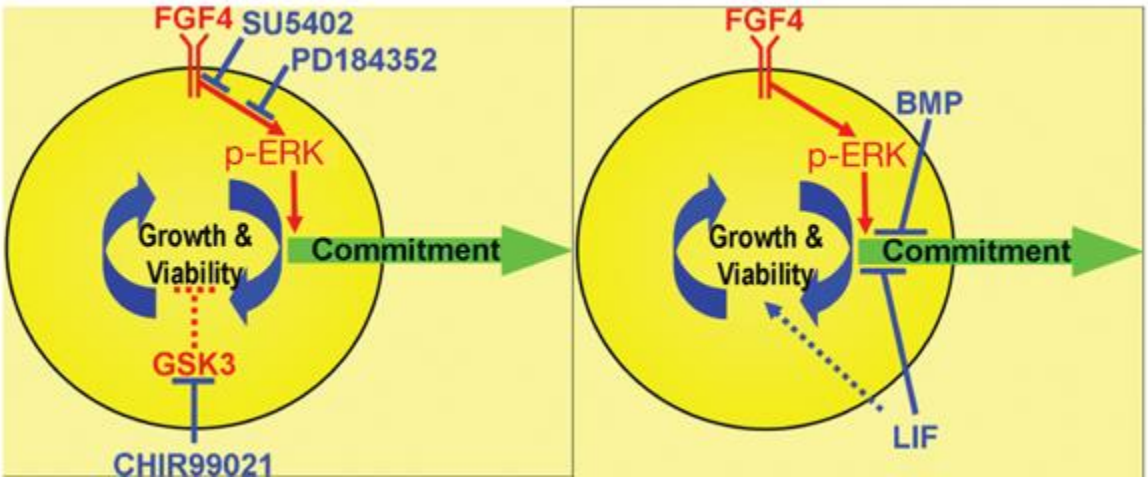


Figure 3 Signalling pathways, which maintain the ground state of pluripotency in ESCs. Self-replication in the pluripotent state can occur when the phospho-ERK pathway and the GSK3 pathway are inhibited by chemical antagonists. Thus, self-renewal is promoted while differentiation is inhibited (Copied from [23]).

5.2.2 Transcription factors

Apart from signalling pathways, pluripotency in embryonic stem cells is also maintained by a particular set of transcription factors that include Oct-4, Sox2, Nanog and Klf4[33-35]. These transcription factors not only bind efficiently to their own promoters but also to the promoters of other genes, which maintain an autologous feedback in the regulatory pluripotent circuit **(Figure 4)** [36-39]. These factors are not only involved in activation of self-renewal, maintenance of pluripotency by binding efficiently to their own promoters but also in inhibition of differentiation into the specialized cells by binding also to the promoters of other genes such as Essrb and Nac1 thereby maintaining an autologous feedback in the regulatory pluripotent circuit

[34-43]. One such example of this kind of binding is the presence of Oct3/4 at any of its binding sites significantly increases the recruitment of Essrb and Dax1 to that particular loci [44]. The core pluripotency factors also recruit RNA polymerase II and other factors which initiate transcription of the self renewal genes in the ESCs. Interestingly, cMyc is known to recruit and increase anti posing protein p-TEFb to overcome abrupt transcription stalling of the genes which may be essential for ES self renewal [44]. The loss of Oct-4 and Sox2 in ESCs leads to pluripotency inhibition and aberrant differentiation into trophectoderm and trophoblast like cells respectively whereas loss of Nanog leads to loss of self renewal potential of the cells *in vitro* and lethal phenotype in embryos through abnormal differentiation into endoderm *in vivo* [33] [34, 35, 45]. The target genes of the pluripotency transcription factors include genes, which maintain pluripotency, and genes, which inhibit the differentiation and lineage commitment such as Essrb, Nanog and Nac1 [34-36, 38, 40-43].

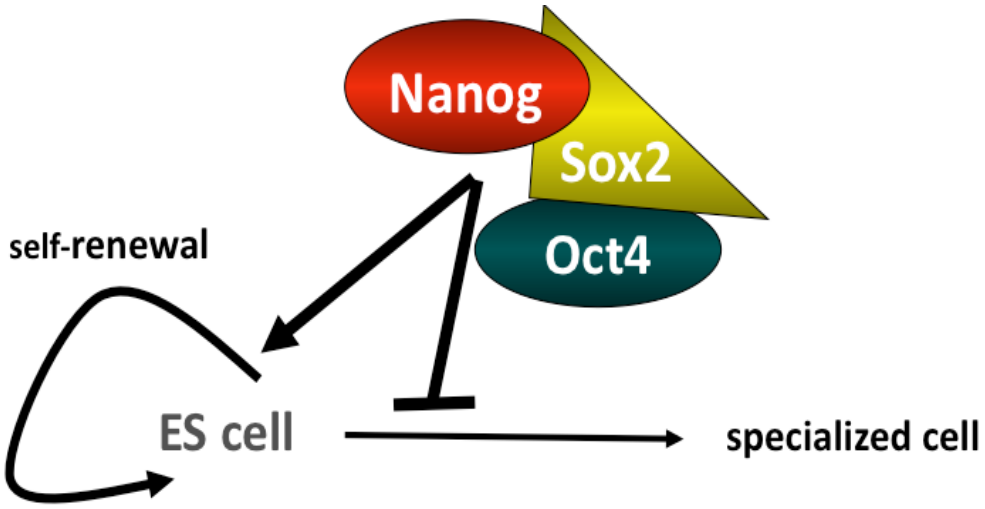


Figure 4 The molecular circuitry of pluripotency. The core pluripotency factors Nanog, Oct-4 and Sox2 are essential for maintaining the pluripotent state of the ESCs by enhancing self-renewal and inhibiting their differentiation into the specialized cells (Copied from [46]).

5.2.3 Epigenetic regulation of pluripotency

An epigenetic hallmark of pluripotency is the X chromosome state. During development, the paternal X chromosome is silenced in the early stages but later in the ICM the inactive X chromosome gets reactivated. However, later in the development towards specific lineages, one X chromosome again shows a random inactivation. Thus the female embryonic stem cells contain two activated X chromosomes in an activated state (XaXa) **(Figure 5)** [47]. This state of the ESCs is usually observed in the naïve pluripotent state, which is usually found in mouse ESCs. ESCs maintained under naïve culture conditions can be demarcated by the presence of two activated X chromosomes [19, 47]. It has been proven that reactivation of inactivated X chromosome is mediated by Nanog [48] which is further maintained with the help of Oct3/4 in ESCs [49].

The process of epigenetic regulation of a cell determines the fate, function, lineage specificity and heterogeneity without any change in its DNA sequences [50-55]. These mechanisms mainly include DNA methylation and different histone modifications like methylation, acetylations at different amino acid residues [56]. In the course of differentiation process some genes need to be silenced whereas others to be activated. This silencing or activation of different relevant genes is mainly accomplished by epigenetic modifications. Differentiation of embryonic stem cells to progenitors or to more differentiated cell stage need an overall change in epigenome. These large scale changes at epigenetic level in turn can affect the gene expression pattern of the respective cell [57].

The chromatin is defined as the combination of DNA and proteins that construct the nucleus of a cell and is usually the target of epigenetic modifications [58]. Chromatin of embryonic stem cells is different from differentiated cells with respect to few but large heterochromatin domains [59-62] with inhibitory modifications at lineage specific genes in euchromatin regions compared to the heterochromatin domains present in the differentiated cells. These inhibitory modifications include acetylation at lysine 9 of histone 3 (H3K9ac) and methylation at lysine 4 of histone 3(H3K4Me) at lineage specific genes [63]. The methylations status of 4^{th} and 27^{th} lysine (K) residue on

histone 3 is of great importance with regards to pluripotency. Methylation at 4^{th} residue marks the activated status of pluripotent genes whereas methylations at residue 27 mark the silent status of these genes [64]. Bivalent domains are defined as the histone 3 methylated on both 4^{th} and 27^{th} residues and these bivalent domains make the genes silent. In embryonic stem cells most of the genes related to differentiation process remain inactive because of the presence of bivalent domains which subsequently converts into other modifications in differentiated cells [56]. The inactivated status of these differentiation related genes is further maintained by repressive functions of pluripotency factors and polycomb repressive complexes. Furthermore PcG proteins catalyze the methylation at the residue 27^{th} [65-68].

Well-documented protein complexes, which regulate pluripotency at chromatin level, are the ATP-dependent chromatin-remodelling complexes. Examples of theses ATPase's include Brahma/Brm, Brg, SNF2H, SNF2L, CHD1 and Mi2-beta which then further assemble into complexes such as BAF and CDH1 respectively. The inactivation of BAF subunits in mouse results in embryonic lethality and abrupt pluripotency which clearly indicates that the BAF complexes are very important for the regulation of pluripotency [69-73].

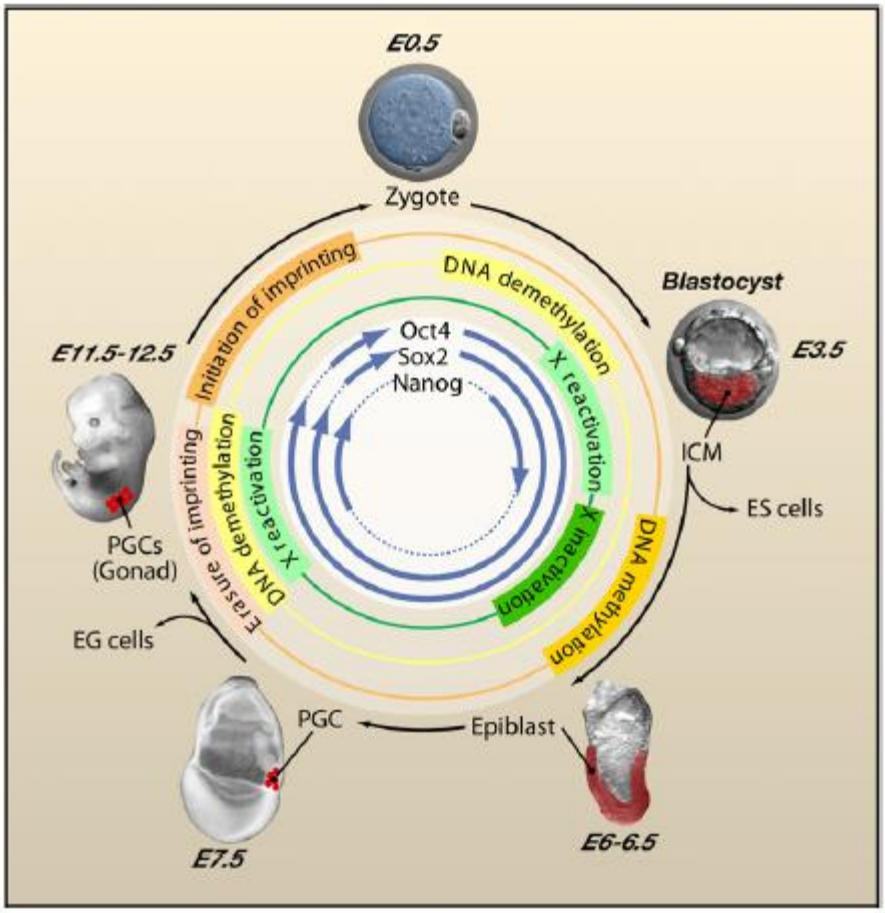


Figure 5 Epigenetic and genetic regulation in the embryo during initial stages of development. The totipotent zygote express maternally imprinted genes such as Oct-4, Nanog and other modulators of pluripotency which further in the blastocysts stage is restricted only in the cells of ICM (red). As the development of the embryo begins, the DNA methylation and demethylation pattern, X inactivation and reactivation status and other modulators decide the fate of the ESCs pushing it towards the differentiation of the cells. (Copied from [74]).

5.3 Cellular reprogramming and different strategies

ESCs are considered as the most suitable cells for the study of human embryonic development, drug screens and toxicology. The futuristic aspect of ESCs also lies in the cell replacement therapies [75]. However, the ethical concerns revolving the isolation of human ESCs are still inevitable. In order to circumvent ethical problems, four different methods were developed to reprogram somatic cells to pluripotent cells

namely (i) somatic cell nuclear transfer (SCNT), (ii) cell-cell fusion with stem cells, (iii) co-culture with stem cell extracts and (iv) induction of defined reprogramming factors [76]. These reprogramming techniques underlined the fact that the epigenome of the somatic cells is not irreversible to its undifferentiated state and can be reversed both *in vivo* and *in vitro*.

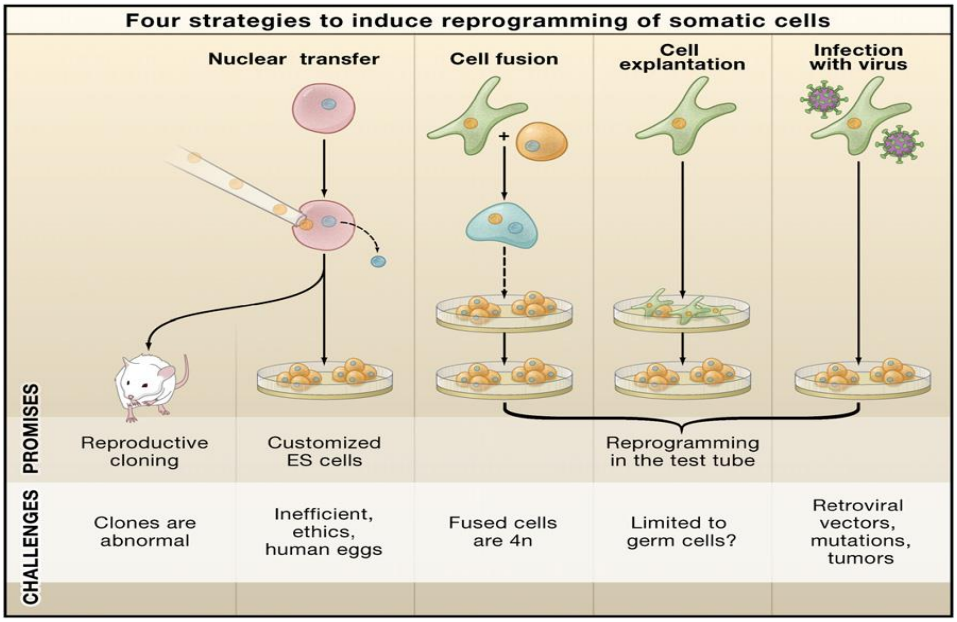


Figure 6 Different strategies for reprogramming of somatic cells. (1) Nuclear transfer involving the transfer of somatic nucleus into enucleated oocyte giving rise to clones *in vivo* and ESCs invitro also known as Somatic cell nuclear transfer (SCNT). (2) Cell fusion of the somatic cells with the ESCs giving rise to hybrids expression ES cell factors. (3) Culture induced cellular explantation of somatic cells which selects for the immortal cell lines which are pluripotent in nature. (4) Generation of induced pluripotent cells by the transduction of defined factors which convert a somatic cell into into a pluripotent state (Adapted from [77]).

5.3.1 Reprogramming by somatic cell nuclear transfer (SCNT)

Somatic cell nuclear transfer (SCNT) is the process of nuclear reprogramming where the nucleus of the somatic cell is transferred into an enucleated and unfertilized oocyte resulting in the reprogramming of the somatic nucleus of oocyte [78]. In 1997, Wilmut and his colleagues pioneered for the first time the *in vitro* nuclear transfer by transplanting the nucleus from a mammary gland cell into an enucleated sheep egg resulting in the first cloned sheep ‘Dolly’ [79]. Following the cloning of Dolly, successful

cloning of other mammalian species such as cow, mouse, goat, cat and pig were performed [80]. During nuclear transfer experiments a very interesting trend has been observed that fully differentiated cells are less susceptible to the reprogramming than the less differentiated or immature cells. For example, when neural stem cells were used to generate the NT-ESCs reprogramming was easier than the reprogramming with neurons [81, 82]. Others study where cloning was done with adult keratinocytes stem cells and transit amplifying keratinocytes, the same trend was again observed providing higher yield of cloning in the case of adult keratinocyte stem cells compared to the differentiated transit amplifying keratinocytes [83]. All these observation point towards the fact that reprogramming efficiency upon nuclear transfer depends on the type of donor nucleus. This dependency occurs due to the reason that the genome of less differentiated cells like stem cells or progenitors is more susceptible to reprogramming than the genome of fully differentiated cells [83].

5.3.2 Reprogramming by cell fusion

Cell fusion is another method used to generate reprogramming in somatic cells. In this method Embryonic Carcinoma (EC), Embryonic Germ cells (EG) or ESCs are fused with a target somatic cell by using chemical called polyethylene glycol (PEG) or physical (electric pulse) fusion agent [84-86]. Evidences that confirm the successful reprogramming include reactivation of epigenetically silenced locus of pluripotency genes like Oct3/4, reversal of X chromosome inactivation etc. Interestingly activation of pluripotency genes occurs not just after fusion but after some cycles of cell division which clearly indicates that this type of reactivation needs replication of DNA [87]. The resulting hybrid cell from this fusion procedure still contain the phenotypic characteristics of parent pluripotent cells thus making it clear that the factors from ESCs also work in new environment and make the pluripotent phenotype dominant over the factors of differentiated target cells [88]. When these hybrids were injected into SCID mice, teratomas containing all germ layers were formed indicating the true pluripotent status of these hybrid cells [89]. Experiments were done with the fusion of target somatic cells, nuclear and cytoplasmic fractions of pluripotent cells. These experiments clearly shown that the reprogramming was only successful when the

nuclear fraction of pluripotent stem were used with the target cells which indicates the necessity of nuclear content to govern the reprogramming and pluripotency [87, 90]. Furthermore when the chromosomes from ES cell origin were deleted from the hybrid cells there was not any effect on reprogramming. This observation clearly showed that only proteins present in pluripotent cells are needed for reprogramming and to maintain pluripotent status of hybrid cells [91]. The major limitation of this method is that the pluripotent hybrids generated from the fusion are tetraploid in nature which limits the clinical application of these reprogrammed hybrids. Studies aimed to overcome this issue by eliminating chromosomes from ES cell origin but again this approach has a risk of high genetic instability [91].

5.3.3 Culture induced reprogramming

It has been observed in variety of studies that cells can acquire status of pluripotency in the course of *in vitro* cultures. For example, bone marrow derived mesenchymal stem cells can give rise to a multipotent cells in long term culture [92]. These cells can further differentiate into different cells and can also contribute to chimera formation upon mouse injection. Another example of such culture induced reprogramming is the generation of multipotent germline stem cells (mGS). It was observed that the mGS when maintained on ESC culture medium show the tendency to form teratomas and chimeras when injected into respective mice [93, 94]. Experiments done with primordial germ cells (PGCs) have also demonstrated that pluripotent cells can be derived from the long term cultures of PGCs in ESCs culture conditions [93]. Further these pluripotent cells were able to form teratomas and to contribute to germ lines in contrast to PGCs which were unipotent cells. Since PGCs are embryonic germ cells that gave rise to ES like cells in culture, adult germ cells were also cultured under the same conditions to produce ES like cells. When cultured under specific growth conditions adult germ cells such as spermatogonial stem cells from adult mouse testis also gave rise to ES like cells [95]. These ES like cells possess all pluripotency phenotypes and contributed successfully to germ line. However, the major imitation with these ES like cells was a different genetic imprinting status compared to the ESCs [95].

5.3.4 Transcription factors mediated reprogramming

In 2006, Takahashi and Yamanaka reprogrammed both mouse embryonic and adult fibroblasts into ES like cells by using viral transduction of four different pluripotency related transcription factor Oct3/4, Sox2, cMyc and Klf4. Those ES like cells have been named as induced pluripotent stem cells (iPSCs). In the initial studies by Yamanaka and Takahashi, 4 genes were selected out of 24 candidate genes as indispensable genes which could revert back a differentiated cell such as fibroblast back to an ESC like pluripotent cell. These four genes were namely Oct3/4, Sox2, c-Myc and Klf4 [96]. Resistance against G148 inserted in the Fbx15 gene provided as the initial criterion to narrow down the candidates to final four. Apart from the initial selection, the other factors which pointed towards the successful reprogramming of the MEFs to iPSCs included endogenous expression of various pluripotency markers [94] inactivated X chromosome [97], contribution to germ line [97-99] , presence of different pluripotent markers [99-101] and teratoma formations.

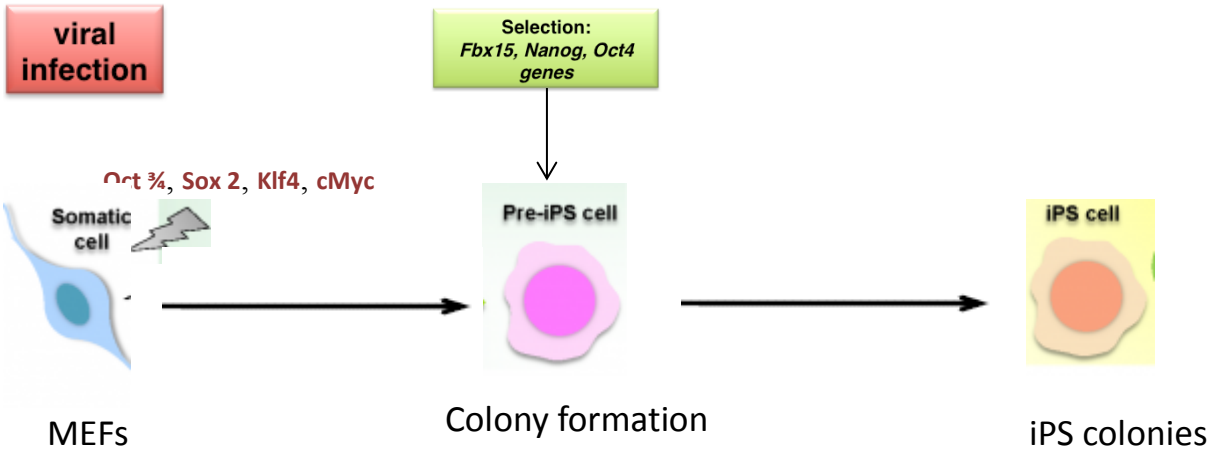


Figure 7 Reprogramming of somatic cells into induced pluripotent stem cells by addition of exogenous pluripotent genes. Transduction of 4 transcription factors Oct3/4, Sox2, Klf4 and c-Myc into fibroblasts converts them back to the pluripotent state from the

differentiated state. The selection scheme by a drug resistance in the Fbx15, Oct3/4 or Nanog genes for the selection of specific iPSCs (Adapted from [77]).

Exogenous expression of these four factors initiate a gradual transformation at both protein and epigenetic level which lead to reprogramming of some infected cells out of total target cell population. The exclusion of Klf4 and c-Myc from the cocktail was sufficient enough for the iPSCs generation however with lower reprogramming efficiencies [102-104]. Recently, it has been shown that other genes such as the orphan nuclear receptor Esrrb (Oestrogen-related receptor beta) functions in conjunction with Oct3/4 and Sox2 to mediate reprogramming in mouse MEFs without exogenous Klf4 and c-Myc. In ESCs, Essrb targets many genes involved in self-renewal and pluripotency, and therefore could act as a general activator-enhancing transcription of common target genes of Oct3/4 and Sox2 during reprogramming [105].

Unlike the reprogramming with SCNT or cell fusion, reprogramming with defined factors take a longer time period to be done as the longer expression of these defined factors needed to active pluripotency genes and to suitable epigenetic modifications [100, 106]. Similar studies have shown that not only somatic cells from liver and gastric epithelia, differentiated B and T cells can be reprogrammed successfully to iPSCs. [107, 108].

However, a major disadvantage with the direct reprogramming is the very low efficiency (max 0.1%) of reprogramming [4, 30, 50, 51]. To overcome this drawback of direct reprogramming secondary systems of reprogramming were developed to allow reprogramming of somatic cells without using direct infection. To this end, doxycycline-inducible reprogramming factors are transduced into somatic cells that upon the induction with doxycycline primary induced pluripotent cells are generated. Injection of the primary iPSCs into blastocysts generates mice in which the daughter cells derived from the primary iPSCs hosts same functional integration of reprogramming factors. Isolation of these transgenic daughter cells and re-stimulation of these cells with doxycycline generates the secondary iPSCs [103, 106]. Reprogramming using secondary system increased the efficiency to 20-40 folds but not more than that as other factors must affect the reprogramming of somatic cells [1].

Other than these methods of reprogramming many other vector systems have been successfully applied to express reprogramming factors. These different vector systems mainly include doxycycline induced lentivirus [103], doxycycline piggyback vectors [109], adenoviruses [110], and episomal vectors [111].

5.4 Molecular mechanism of *in vitro* reprogramming

One of the major questions in the field of cellular reprogramming that remains to be addressed is that the gradual course of the reprogramming procedure culminating in low reprogramming efficiencies. To explore more into the factors affecting reprogramming, a whole set of factors such as genetic differences between target cells, need of additional expression of genes after ectopic expression of reprogramming factors and presence of specific population within a target cell population making it more susceptible to reprogramming should be studied [4].

To address these limitations, a set of experiment to test whether the homologs of core reprogramming factors can play role in the *in vitro* reprogramming, 13 different but related transcription factors for reprogramming were tested in 2008. Interestingly, it was observed that none of the homologs of Oct3/4 could successfully reprogram the cells but the related factors to Sox2 and Klf4 could reprogram the cells successfully. The abolition of c-Myc from the reprogramming cocktail lead to the lesser generation of tumours in mice injected with iPSCs generated using the 4 factor approach indicating the not so essential role of c-Myc in reprogramming [102, 112]. Some other factors such as Esrrb and Nr5a2 could successfully replace Klf4 and Oct3/4 in 4 factor reprogramming method [105, 113].

Another possible explanation for the slow and less reprogramming could be the stochiometry of different signalling processes, protein activation and epigenetic program in the different target cells. Cells from same population can be heterogeneous at the genetic level thereby varying the expression of 4 factors that can lead to reprogramming of particular subpopulation of somatic cells expressing similar levels of reprogramming factors. It has been observed that the different markers used

for the analysis of reprogramming like nanog, SSEA1, Alkaline phosphate are activated at different time points [41, 114]. All these phenotypes are correlated with the epigenetic changes and gene expression patterns which occur at different time points in cells with different genetic backgrounds. *In vitro* reprogramming can also affected by culture environment as growth factors, cytokines and other factors which are added to the medium can alter the signalling processes at molecular level. It has been observed that when some particular signalling pathways like Wnt signalling were increased the reprogramming efficiency also increased [115]. During *in vitro* reprogramming these epigenetic changes can take time making the whole reprogramming procedure gradual and time consuming. These epigenetic changes include reversion of heterochromatin silencing at Oct3/4 promoter, and demethylation at pluripotency gene locus. It has been observed that regions which were hyper methylated somatic cells got demethylated in iPSCs [97-99].

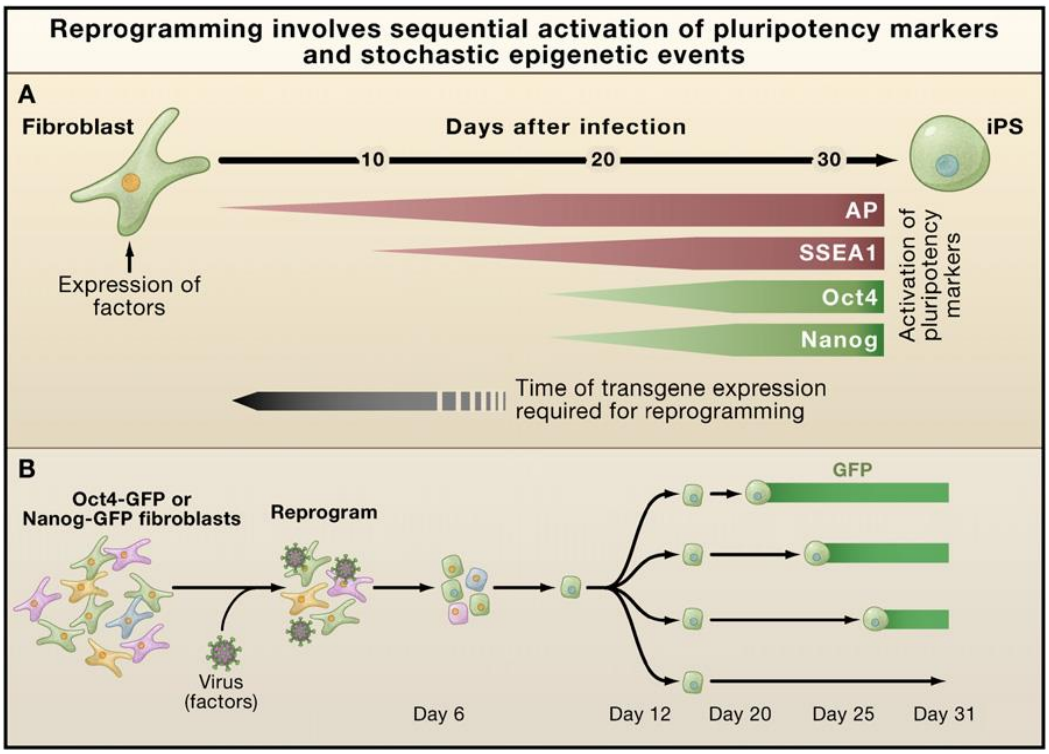


Figure 8 Kinetics of reprogramming. (A) The transduced iPSCs colonies are selected on the basis of their pluripotency marker expression, SSEA1 expression and their positivity to alkaline phosphatase (AP). (B) Reprogramming is a stochastic process with different clone showing the activation of Oct3/4 EGFP reporter at different time points (Adapted from [77]).

To bypass viral reprogramming several attempts were made to deliver these reprogramming factors into target cells by non-integrating viruses, mRNA transfection, small molecules and/or recombinant proteins [101, 116-118]. The major drawback of generation of iPSCs by these methods was the severely reduced overall reprogramming efficiencies compared to virus mediated reprogramming. To circumvent the insertion of the reprogramming cassette in the genome, a recombinant Cre recombinase approach can be used to excise the exogenous DNA from the genome [109, 119-121].

5.5 Scientific opportunities given by pluripotent stem cells

Induced pluripotent stem cells provide various therapeutic opportunities including cell therapy, disease modelling and drug development.

5.5.1 Cell therapy using induced pluripotent stem cells

The major problems which occur during organ transplantation include very less availability of matched donors and the side effects of immunosuppressive drugs. These problems can be avoided by using induced pluripotent stem cells as genetically matched differentiated tissues and organ can be prepared with iPSCs. With the help of modern gene targeting approaches iPSCs can be used as the potential therapeutic agent to treat various genetic diseases [122]. One of the prominent example of this approach is the treatment of sickle cell anaemia in animal model where iPSCs from the skin cell of the model mouse were prepared, target mutation was repaired and then differentiated blood progenitors were transplanted back in the mouse to treat the disease [122]. iPSCs derived endothelial progenitor cells were also tried to treat haemophilia A mouse model and maybe also tissue repair [123].

5.5.2 Disease modelling and drug development using iPSCs

In this approach iPSCs are generated from the patient cells and then these iPSCs are differentiated to affected cell types of a particular disease for example affected cells of diabetes or Parkinson's disease. Furthermore these differentiated affected cell types can be used as disease models for various purposes like drug screening or studying the disease causing mechanisms. iPSCs can also be used to study the early stages of a disease which is detectable only in advance stages as patient specific iPSCs can be differentiated into disease affected cells to study different stages of disease progression. Thus, iPSCs have proven a significant tool to elucidate pathophysiological mechanisms in various diseases such as diabetes, blood disorders, defined neurological disorders and genetic liver disease [124-126]. iPSCs enable the dissection of monogenic human disease [127] mechanisms as well as mechanisms of genetically complex human disorders such as schizophrenia [128]. This opens promising perspectives both for the screening of innovative, "druggable" targets [129] and *ex vivo* gene targeting therapies [127] [130].

5.6 Drawbacks of the iPS cell technique

ESCs can differentiate into almost all cell types both *in vitro* and *in vivo* and this unique property of pluripotency makes them the most valuable tool for understanding the mechanisms underlying various diseases and as also the potential cure for the most aggravating diseases [131]. However, the ethical issues surrounding the isolation of ESCs from human embryos are big drawback in use of ESCs as a therapeutic option. The discovery of generation of Induced Pluripotent Stem cells (iPSCs) marked a revolutionary milestone not only in developmental biology but also for new therapeutic approaches as these cells fulfilled all the prerequisites of ESCs but partly circumvented the ethical and immunogenic limitations of the therapeutic use of ESCs [132]. Nevertheless, a trivial hitch of this technology for the use in regenerative medicine is the several genomic integrations of the lenti or retroviral over expression systems which can lead to the potential risk of insertional mutagenesis leading cellular transformations and tumour formation [133]. The other constraint in practical use of this technique is the very low reprogramming efficiencies of usually <1% of the target cells and the longer duration of the reprogramming process itself [132, 134].

It has been hypothesized that the cell type of origin influences the reprogramming process by favouring a respected lineage [135, 136]. It has already been shown that iPSCs generated from different donor cell types have different epigenetic and transcriptional make up [137]. iPSCs generated from fibroblasts, hematopoietic and muscle system cells show differences in their differentiation potential because it is believed that these iPSCs retain the epigenetic memory of their somatic cell origin [137]. This retention of somatic cell epigenetic memory influences the selection of certain type of donor cells for iPSCs generation.

Epigenetic memory also plays an important role in some cases to make certain cell types suitable candidate for iPSCs generation. For example somatic cells like NSCs and dermal papilla cells are known to maintain some epigenetic characteristics similar to ESCs at Sox2 and Nanog locus [138] [139]. This kind of epigenetic patterning

makes these cells more susceptible towards reprogramming as they need less epigenetic re-shift to become pluripotent. Thus they can be reprogrammed using less number of ectopically expressed reprogramming factors [138] [139].

Moreover, the reprogramming kinetics differs amongst the various cell types in the same microenvironment. Thus, the identification of certain subpopulations in the same tissue origin which are more open towards the process of reprogramming could benefit the community by helping to generate fast and safer iPSCs cells which show unbiased differentiation capacity.

6 Aim of the thesis

It has been already reported that stem/progenitor cells from skeletal muscle [140], brain [141], and hematopoietic system are superior in cellular reprogramming compared to their differentiated counterparts. However, it was unclear whether just the differentiation stage of the cells affects reprogramming efficiency or whether there are some cell intrinsic factors that also play a role in the regulation of this phenomenon. Adult stem cells share common features with their pluripotent counterparts, such as extended differentiation potential and self-renewal [142, 143]. Along these lines, it was shown that epigenetic modifications in adult or tissue specific stem cells more closely resemble those in ESCs compared to somatic cells [144-148]. This is reflected by an enhanced reprogramming efficiency of tissue stem cells.

Thus, the aim of this thesis were

(i) To study whether different somatic cell populations (progenitor cells versus differentiated liver cells) isolated from parenchymal organ such as murine liver show a hierarchy in their reprogramming efficiencies and

(ii) Whether these higher reprogramming efficiencies in the progenitors are independent of expression of endogenous pluripotent genes which are present in the reprogramming cocktail.

(iii) Are these reprogramming efficiencies independent of proliferation?

(iv) Whether cell intrinsic factors such as chromatin remodelling factors enhance the reprogramming efficiency of cells.

To address these questions, we isolated liver progenitor cells (LPCs), non- LPC, hepatocytes and cells from whole liver and subjected them to reprogramming in same cultural conditions to test their ability and capacity of reprogramming.

7 Materials and Methods

7.1 Materials:

Laboratory Equipment	Supplier
Cell Culture Incubator	Sanyo
Cell Culture Laminar Flow Bench	Biowizard
Cell viability analyzer Vi-Cell XR	Beckman Coulter
Avanti Centrifuge	Beckman Coulter
Allegra Centrifuge	Beckman Coulter
Chemiluminescence	Vilber Lourmat
Table Centrifuge	Thermo Fischer, Eppendorf
FACSAriaTM II	BD Biosciences
LSR II	BD Biosciences

PCR Cycler	SensoQuest
7300 Real Time PCR System	Applied Biosystems
Microscope DM 5000B	Leica
Swinging Bucket Ultracentrifuge	Beckman Coulter
Agarose Gel Electrophoresis Apparatus	BioRad
Gel Documentation System	Vilber Lourmat
Nanophotometer	Implen
Vortex	Heidolph
Water Bath	GFL

7.2 Software

Leica LAS AF	Leica Microsystems 28
Cell Quest Pro	Becton Dickinson Biosciences
Flowjo 7.2.2 .5	Tree Star
GraphPad Prism 5	GraphPad Software
Microsoft Office 2011	Microsoft

7.3 Chemicals:

Chemical	Supplier
Agarose	Sigma
Bromophenol Blue	Serva
Diethylpyrocarbonate (DEPC)	Sigma
dNTPs	Bioline
DAPI	Vectashield
EDTA (N, N; N', N' – Ethylenediaminotetracetic acid)	Calbiochem

Ethanol	Sigma
Ethidium bromide	Merck
Glycerol	Sigma
Foetal Bovine Serum	Lonza
Goat Serum	Abcam
HEPES	Sigma
Hydrochloric acid	Merck
Isopropanol	Sigma
β-Mercaptoethanol	Sigma

PBS (Phosphate Buffered Saline)	Gibco
RNAse Away	Invitrogen
Tris (Tris-(hydroxymethyl)-aminomethane)	Sigma
Triton-X-100	Sigma
Tween 20	Sigma
Xylol	Merck
1 kb plus DNA ladder	Invitrogen
GoTaq DNA Polymerase	Promega
Restriction Endonuclease	NEB
RNAse H	Invitrogen

Superscript III Reverse Transcriptase	Invitrogen
T4 Polynucleotide Kinase	NEB

7.4 Cell culture reagents

DMEM	Sigma
Knockout DMEM optimized for ESCs	Gibco
Foetal Bovine Serum (FBS)	Lonza
Foetal Bovine Serum ES cell pretested	PAA
Trypsin/EDTA with Hanks salt	Millipore
Penicillin/Streptomycin	Millipore
Leukemia Inhibitory Factor (LIF)	ESGRO-Millipore
Non Essential Amino Acids (NEAA)	Gibco
Sodium Pyruvate	Gibco
Glutamax	Gibco

7.5 Animal Studies

The following mice models were used in the study:

1) **B6;CBA-Tg(Pou5f1-EGFP)2Mnn/J (Pou-eGFP):** These mice are homozygous for the transgene insert and express Enhanced green fluorescent protein under the control of the Oct ¾ promoter and a distal enhancer.
2) **Crl:CFW (Swiss Webster mice):** These mice are albino mice carrying black agouti behind its albino gene.

7.6 Antibodies

Antibody	Dilution	Company
Oct 4	1:100	Santacruz
SSEA1(ascites fluid)	1:500	Developmental hybridoma
α-actinin	1:150	Sigma
ß3-tubulin	1:250	Santacruz
Nestin	1:100	Santacruz
CK19	1:50	Santacruz
Albumin	1:100	Bethyl Diagnostics
Alexa Fluor® 488	1:500	Invitrogen
Alexa Fluor® 568	1:500	Invitrogen
Alexa Fluor® 647	1:500	Invitrogen
Alexadye-conjugated	1:400	Invitrogen

phalloidin		
CD45	1:10	BD Biosciences
Ter119	1:10	BD Biosciences
c-Kit	1:10	BD Biosciences
Sca1	1:10	BD Biosciences
CD13	3µl	BD Biosciences
CD133	1.5µl	BD Biosciences
CD49f	20 µl	BD Biosciences
Dlk1	20 µl	BD Biosciences

7.7 Primers

Name of the Primer	**Company**
Hmbs	Qiagen
Nanog	Biomers
Oct3/4	Biomers
Nkx 2.5	Qiagen
Sox2	Biomers
Klf4	Biomers
c-Myc	Biomers
Rex1	Biomers
Nestin	Qiagen

Alphafeto protein	Qiagen
Smarcca	Qiagen
Smarca4	Qiagen
Myh6	Qiagen
c-Kit	Biomers
ß3-Tubulin	Biomers
Brachyury	Biomers
Albumin	Qiagen
Gata-4	Qiagen

7.8 Isolation of liver progenitor cells

For the isolation of foetal liver cells, whole foetal livers from 8-10 embryos of E14.5 Pou-EGFP mice were removed and then minced in PBS into fine pieces. Liver cells were isolated by incubating the minced livers for 15 minutes in 0.05% collagenase solution at 37°C in water bath for mechanical dissociation. The cells were washed with HBSS medium and PBS and then stained for 1hr with the FACS antibodies specific for surface markers present on liver progenitor cells [149]. The antibody combination for FACS sorting different cell populations was as follows:

- LPC (marker Positive): CD45- Ter119- cKit- CD13+ CD133+ Dlk+.
- Non-LPC (marker negative): CD45- Ter119- cKit- CD13- CD133- Dlk-.

For the isolation of adult liver LPCs, the livers from the adult Pou-EGFP mice were perfused with 1-3mls of pre-perfusion buffer (HBSS with 10 mM HEPES and 0,5 mM EGTA) for 3 minutes followed by 1-3mls of collagenase buffer (HBSS with 5 mM

$CaCl_2$ and 10 mM HEPES) and 0,4 mg/ml Collagenase IV for 5-8 minutes. The perfused livers were cut into small pieces and re-suspended in 20ml DMEM containing 10% FBS and 1% Pen/strep/Glut and triturated by pipetting. The cell suspension was filtered through a cell strainer and then centrifuged 50g for 1min at 4°C to separate the non- parenchymal cell solution containing stem/progenitor cells. The supernatant contains the stem/progenitor cells whereas the pellet has mature hepatocytes. After several centrifugation steps of the supernatant, the cells were then re-suspended in 25% Percoll and spun down to remove all the hepatocytes if any left. The supernatant containing the progenitor cells was collected and spun down again to remove any remnants of Percoll. The cell pellet was resuspended in 50µls of 2% FBS/PBS and stained with FACS antibodies for 1 hr and sorted on a BD FACS Aria-II flow cytometer.

For Adult LPCs, the following antibody combination was used:

(CD45- Ter119- cKit- Sca1- CD13+ CD133+ CD49f+).

7.9 Generation of Mouse Embryonic Fibroblasts (MEFs):

Young Pou-eGFP (8-14 weeks) mice were mated and positive plug check was reported to identify pregnant females. 13 days after a positive plug check, the pregnant female mice were sacrificed and the embryos were freshly harvested in 10 cm dish covered with PBS. The embryos were separated from both the placental and maternal tissues and transferred to a new dish with fresh PBS. Next the head of the embryo was cut and all the internal organs such as heart and liver were removed using a sterile forceps. To isolate the cells, the body of the embryo was minced in 2mls of 0.25% Trypsin-EDTA (Millipore) with sterile scissors in a 6 well plate to get a cell suspension. The plate with the minced embryos was incubated at 37°C for 3-5 minutes for the activation of trypsin. After 5 minutes, the trypsin activity was quenched using 3 mls of MEF medium consisting of DMEM with 15% FBS+1% P/S+ 1% ß-mercaptoethanol, 1%Sodium pyruvate, 1% glutamax and 1% Non-Essential Amino Acids (NEAA). The mixture was then pipetted 10-20X to break up all the large tissue parts if any. The whole cell suspension was then transferred to a new 10 cm dish

coated with 0.2% gelatine and fresh medium and incubated overnight at 37%. After 12 hrs, the medium was aspirated and the cells were washed 3X with PBS to remove any excess tissue parts. 10 mls of fresh medium was added on the cells and incubated again till the plate was confluent. On reaching confluence, the cells were split in the ratio of 1:5 and then harvested and frozen down in liquid nitrogen for further usage [150].

7.10 Generation lentiviruses:

$5x10^6$ lenti-X cells (Clonetech) were seeded on collagen coated 10cm dishes. After 12 hrs, a mixture of 15µg of either polycistronic StemCCA-4F (OKSM) or StemCCA-3F (OKS) vectors [119, 120], 10µg of Pax2 (packaging vector) and 5µg of MD2 (envelope vector) was prepared and 2M calcium was added to it. Next 700µls of HBS was added drop wise to this mixture while bubbling the mixture simultaneously. The transfection mixture was incubated at RT for 20 minutes and then added to the previously seeded lenti x cells for transfection. Medium was changed after 8-10 hrs after washing once with PBS for removing the transfection reagent. Lentiviral supernatant was collected after 12 hrs, 24 hrs and 48 hrs of changing the medium. The supernatant was filtered through 0.45 µm filter and concentrated by spinning at 25,000 rpm at 4°C for 90 minutes using a Beckman Ultracentrifuge. The viral particles were diluted 1000-fold by resuspending in DMEM, alliquoted as 50µls per tube and stored immediately at -80°C for further usage. The protocol was performed according to the manufacturer's guidelines (Clonetech).

7.11 Reprogramming of different liver cell subpopulations from foetal and adult mouse liver and iPSCs cell culture

Adult and foetal progenitor LPCs and non-LPCs were sorted on collagen coated 12 well plates (2000 cells/well) after staining as per the protocol mentioned in 4.5. Same number of hepatocytes were isolated and seeded on collagen coated 12 well plates simultaneously. One day after seeding, the cells were infected with equal amount of

reprogramming virus containing OKSM or OKS. One day after the infection, Mitomycin C treated mouse embryonic fibroblasts were added as feeders and subsequently culture conditions were switched to ESC culture conditions. ES cell medium (KO-DMEM+ 15% ES-FBS+ 1% LIF+ 1% ß-ME+ 1% Sodium pyruvate+ 1% Glutamax+ 1'% NEAA+ 1% P/S) was changed after every two days. Cultures were observed daily and colony formation was documented using a Leica microscope based on the eGFP expression due to the expression of Oct3/4 promoter. On day 10, cultures were fixed and stained for alkaline phosphatase (AP)-expression as AP is considered as a marker for ESCs. Arising iPSCs clones from either adult or foetal non-LPC or LPCs were randomly picked based on typical ES cell like morphology at day 10 of reprogramming and further expanded. Established iPSCs lines were cultivated in the undifferentiated state on Mitomycin C treated MEFs in DMEM containing 15% FCS (ES-cell qualified) and LIF.

7.12 Alkaline phosphatase staining

For alkaline phosphatise (AP) staining, the cells were washed with 1xPBS to remove all the traces of medium and were fixed with 4% PFA for 2 minutes. The cells were then washed with PBS and the AP staining solution (10ml NTM buffer+ 333µls NBT+ 35µls BCIP) was added. The cells were kept in dark with the working solution till the blue colour developed on the colonies. Later the cells were washed with PBS and the colonies were counted under the microscope and each colony was investigated for Oct3/4-EGFP positivity [1] .

7.13 Calculation of reprogramming efficiency

To calculate reprogramming efficiency, separate wells of the same experimental liver cell preparation of both LPCs and non-LPCs were infected with 3F reprogramming viruses containing StemCCA-mCherry viral particles. 3 days after infection, 30 visual fields were counted for mCherry-positive cells in order to determine the transduction efficiency in LPCs and non-LPCs. At day 10, the plate was fixed with 4% PFA and

stained with AP solution to identify reprogrammed cells. Finally, reprogramming efficiency was calculated by dividing the number of AP-positive and/or EGFP-positive colonies by the number of initially infected cells (transduction efficiency multiplied with number of seeded cells).

7.14 Teratoma formation

LPC derived iPSCs from both foetal and adult origin were grown under standard ES cell conditions on inactivated MEFs. One million cells were sorted based on the Oct3/4-EGFP expression and injected subcutaneously in both the sides of NOD.Cg-Prkdc SCID Il2rg mice. Transplanted mice were sacrificed after 4 weeks and tumours were explanted and subsequently embedded for further analysis.

7.15 Haematoxylin and Eosin staining on teratoma sections

The LPC derived teratomas were fixed in 4% PFA for overnight after harvesting from the mice and embedded in a paraffin block. The blocks were sectioned using a microtome and collected on a slide. The slide was left to dry overnight at 4°C. For H and E staining, the slides were de paraffinised by treating the slide 3 times with xylene for 3 minutes each and then rehydrated by gradual ethanol treatment. The sections were incubated for 3 minutes each in 100% ethanol, 95% ethanol and 70% ethanol and then washed thoroughly with distilled water to remove any ethanol residues. The sections were then incubated in haematoxylin for 6 minutes and then washed thoroughly in distilled water to remove the excess staining. The sections were then counterstained with eosin by dipping the slide in the eosin solution for 15 seconds. Excess staining was then washed in distilled water and the sections were dehydrated again with increasing dilutions of ethanol. The sections were subjected to 70%, 95% and 100% ethanol for 3 minutes each and then again cleared using xylene for 5 minutes. The sections were covered with cover slip using Entellen mounting medium (Millipore) and then subjected to microscopy for teratoma assessment [151].

7.16 *In vitro* differentiation of iPSCs

In vitro differentiation of iPSCs was carried out according to the standard hanging drop method. 400 cells of LPC derived iPSCs in 20 µl drop of differentiation medium containing Iscove's modified Dulbecco's medium supplemented with 10% FCS, 1% GlutaMax, 1% NEAA and freshly prepared monothioglycerol (final concentration 450 µM) were seeded on the inside of the lids of the Petri plates. The bottom of the plate was filled with 10 ml of PBS. The plate containing hanging drops were left undisturbed for 2 days to form embryoid bodies (EBs). After 2 days, the EBs were flushed and cultured in suspension in differentiation media on 6cm plates. The 4 days old EBs were then plated on gelatine coated 6 well plates or on cover slip in differentiation medium and collected as per the requirement of the experiment and assayed at specific time points [152].

7.17 Hepatic differentiation of iPSCs

For hepatic differentiation of MEF-iPSCs (passage 15), 3F-adult-LPC-iPSCs (passage 20) and 3F-hepatocyte-iPSCs (passage 16), a modified cytokine based approach was used. The iPSCs were seeded on a monolayer on gelatine coated 6 well plate without the addition of feeder layer. The cells were then subjected to differentiation medium containing advanced DMEM/F12 + 50 ng/ml Activin A for two days to induced primitive endoderm formation followed with the addition of 10ng/ml bFGF and 50 ng/ml BMP4 to induce hepatic progenitor like cells. After day 10 the medium was replaced with foetal liver-conditioned medium supplemented with 10 ng/ ml EGF, 20 ng/ml HGF, dexamethason, and 10 ng/ml oncostatin M to induce maturation into hepatocytes. The differentiated hepatocytes were identified by the expression of albumin and cytokeratin 19 on protein levels as well as functional tests like Cypa11 activity [153].

7.18 Measurement of cytochrome activity

Activation of cytochrome P450 subtype 1A1 was assessed by GoScript P450-Glo™ CYP3A4 kit as per manufacturers protocol (Promega). In a 96 well plate, 50 µls of

hepatocyte like cells from LPC derived iPSCs were added from a stock solution of 2 million cells/ml. To this, 50µls of 2X Luciferin-IPA/2X test compounds were added and the plate was incubated at 37°C for 15 minutes for Luciferin-IPA reactions. After 15 minutes, 100 µls of Luciferin detection reagent was added to each well and the sample was thoroughly mixed. The sample was then equilibrated at room temperature for 15-20 minutes and then the luminescence was measured using a Tecan machine.

7.19 RNA isolation

The required cells were harvested in lysis buffer and total RNA was isolated using Qiagen Minikit (74104) according to the manufacturer's protocol. The harvested cell lysate was loaded on QIAshredder to homogenize and then the flow through was loaded on mini columns. The mini columns were washed once with RW1 buffer and twice with RPE buffer and spun down at full speed for 15 seconds. The RNA was then eluted using the elution buffer supplied in the kit and stored at -80°C. RNA concentration was measured with a Nano photometer or Bio analyzer. A 260/280 and 230/260 ratio of more than 1.8 absorbance units were considered a good RNA preparation.

7.20 cDNA synthesis

cDNA synthesis was performed using GoScript Reverse Transcription Kit according to the manusfacturer's protocol (Promega). Briefly, 400ng of RNA and 50 units of dNTPs were incubated together at 70°C for 10 mins. A mixture of 5 x GoScript transcriptase buffers, PCR nucleotide mix, 2.5 mM $MgCl_2$, 0.5 units of RNAsin H and RT enzyme were added to the sample after 10 mins. The RT reaction was carried out at 25°C for 10 mins, 50°C for 60 mins followed by a termination step at 75°C for 10 mins. The cDNA was diluted in the ratio of 1:10 and stored at -20°C for further use.

7.21 Real time PCR

Relative gene expression analysis was done with the help of quantitative real time PCR method using syber green (SYBR) probes. 200 ng of cDNA and 1 µmol of both sense and anti sense primers were amplified per assay reaction and gene expression was measured relative to GAPDH or HMBS as an internal control. Each individual sample was measured in triplicates and all the experiments were repeated 2-3 times. For SYBR (iTaqTM SYBR green super mix with ROX, BIO-RAD, 172-5850) based RT-PCR, melting curve analysis was carried out to examine primer specificity. The amplification was analyzed by measuring the binding of the fluorescence dye SYBR green to the minor groove of double stranded DNA. Standard RT-PCR conditions with 10 min of reverse transcription at 50°C, 45 cycles of denaturation at 94°C for 10 s each and 60°C were used.

7.22 Proliferation assays

The measurement of cell proliferation in the liver cells was reported using two different techniques; Propidium Iodide (PI) staining and Bromodioxyuridine (BrDU) labeling. For both the analysis, the LPCs and the non-LPCs were freshly isolated from the foetal liver as described above and sorted based on their surface markers. The cells were plated without feeder and were subjected to the OKSM virus. For PI staining, the cells were harvested after four days of infection. The cells were then fixed by adding 80% chilled ethanol drop wise to the cells. After 30 mins, the cells were resuspended in 0.1% sodium citrate/triton-x-100 buffer containing 50 µg/ml propidium iodide and incubated for 1 hr at room temperature. The cells were analyzed by FACS LSR II to assess different cell cycle stages. For BrDU assay, the cells were pulsed with BrDU for six hours after four days of transduction with the OKSM virus. The cells were fixed with 70% ethanol at 4°C for 30 minutes. The cells were then permeabilised with 2N HCl containing 0.5% Triton X-100 and incubated at room temperature for 30 minutes. The cells were spun down at 500 g for 10 minutes and resuspended in 0.1 M $Na_2B_4O_7$, pH 8.5. The cells were washed using PBS containing 0.05% Tween20. After washing, the

cells were stained with anti-BrDU antibody FITC (BD, 1:200) and number of cells positive for BrDU was counted by 3 wells of a 12 well plate.

7.23 Mathematical curve fitting analysis

For calculating the influence of cell proliferation rates on the reprogramming in different liver cell populations, mathematical curve fitting analysis of cellular reprogramming in LPCs and non-LPCs was done. All experimental data were fitted (R version 2.11, function "nls") with sigmoidal functions of the form: f (d) = 1 / [1 + exp (-a x + b)]. Parameter values were: a = 0.372, b=2.311 (LPCs) and a=0.1116, b=7.7868 (non-LPCs). The latency for reaching the same plateau was calculated by determining the inflection point of sigmoidal functions fitting each data set. Inflection points were calculated to be at day 6.21(LPCs) and day 69.77(non-LPCs) [1].

7.24 Immunofluorescence and FACS staining

For intra-nuclear staining, the cells were harvested with trypsin/EDTA and then fixed with 4% PFA for 30 mins. Following fixation, the cells were permeabilised with saponin and then stained with primary antibodies (1:200) diluted in saponin/PBS for 1 hr at 4°C followed by 3 washes with PBS. The cells were then stained with an appropriate secondary antibodies diluted in saponin/PBS (1:500) and then analyzed with FACS to check for the percentage of positive cells.

For surface marker staining, the cells were seeded on gelatin coated coverslips and then harvested with trypsin/EDTA and the suitable primary antibodies (1:200) were added to the cells. The cells were incubated for 1 hr at 4°C in the primary antibody followed by washing with the PBS. The cells were then subjected to secondary antibodies (1:500) diluted in PBS and the cells were again incubated at 4°C for 1 hr. After washing off the secondary antibody, DAPI (1:10,000) was added to the cells and the images were documented using a Leica fluorescence microscope.

7.25 Chimera generation

For blastocyst injections, female BCF1 mice were super ovulated by intraperitoneal injection of PMSG and HCG and then mated to ICR stud males. The vaginal plug was checked every 24h after HCG administration. Two days later, 8-16 cells were isolated from female oviducts. Isolated embryos were cultured in KSOM media *in vitro*. 10-15 iPSCs were injected into *in vivo* fertilized blastocysts. Recipient female mice were mated with vasectomised mice and embryos transferred at dpc 2.5 to pseudo-pregnant recipient females. The generation of chimeras were performed in Münster in the laboratory of Prof. Dr. Hans Schöler by Dr. Jin Young Joo.

7.26 Transcriptome analysis

Gene expression evaluation was carried out with RNA isolated from freshly isolated foetal liver cells (LPC and non-LPC) and LPC-iPSCs using the Agilent Whole Mouse Genome Oligo Microarray Kit (4x44K microarray kit G4122F (LPC and non-LPC) and 4x44K V2 microarray kit G4846A (LPC-iPSCs), Agilent Technologies, Santa Clara, CA, USA, http://www.home.agilent.com). A sample included around 150.000 cells isolated from a pool of several foetal livers. Cy3-CTP labeled cRNA was produced from 300 ng of total RNA using the Agilent Quick Amp Labeling Kit. After labeling and cRNA purification, cRNA was quantified using the Nano Drop ND-1000 UV-VIS Spectrophotometer. Cy3 labeled cRNA (1.65 µg) was hybridized per individual array for 17 h at 65 °C (10 r.p.m.). After hybridization, arrays were washed consecutively with Agilent Gene Expression Wash Buffers one and two, and acetonitrile for 1 min each. Slides were scanned immediately following washing in the Agilent Scanner using Scan Control 7.0 software. Expression data were extracted using the Feature Extraction software (Agilent). Preprocessing of expression data was performed according to Agilent′s proposed standard workflow. Using 5 flags (gIsPosAndSignif, gIsFeatNonUnifOL, gIsWellAboveBG, gIsSaturated, gIsFeatPopnOL) from the Feature Extraction software, probes were labeled as detected, not detected, or compromised. Gene expression levels were background corrected and signals for duplicated probes

were summarized by geometric mean of non-compromised probes. After log2 transformation, percentile shift normalization at the 75% level was performed. For comparison between data from different Agilent array, only probe sets present on both platforms were used (12088 probe sets). All computations were done using the R statistical software framework (http://www.R-project.org).

Data sets were compared to sets from GEO (http://www.ncbi.nlm.nig.gov/geo): GSM648512_K-G6-MEF.txt: MEF-IPSCS.1; GSM648513_K-H8-MEF.txt: MEF-IPSCS.2; GSM648514_K-H10-MEF.txt: MEF-IPSCS.3; GSM648515_4F-MEF.txt: MEF-IPSCS.4; GSM648516_MEF.txt: MEF; GSM585266.txt: ES.1; GSM585267.txt ES.2; GSM721145_251486829226_1.txt: UT_en.1; GSM721146_251486829227_3.txt: UT_en.2; GSM721147_251486829227_4.txt: UT_en.3; GSM721148_251486829228_2.txt: UT_en.4; GSM721149_251486829229_3.txt: UT_en.5; GSM721150_251486829225_1.txt: UT_dep.1; GSM721151_251486829226_3.txt: UT_dep.2; GSM721152_251486829228_4.txt: UT_dep.3

Own data was uploaded to GEO: IPSCS_1_252665511949_1_1.txt: IPSCS.1; IPSCS_2_252665511949_1_2.txt: IPSCS.2; IPSCS_3_252665511949_1_3.txt: IPSCS.3; LPC_1_251486822913_1_1.txt: LPC.1; LPC_2_251486822913_1_3.txt: LPC.2; LPC_3_251486822914_1_1.txt: LPC.3; LPC_4_251486822914_1_3.txt: LPC.4; nonLPC_1_251486822913_1_2.txt: nonLPC.1; nonLPC_2_251486822913_1_4.txt: nonLPC.2; nonLPC_3_251486822914_1_2.txt: LPC.3; nonLPC_4_251486822914_1_4.txt: LPC.4.

Only probe sets with a fold-change > 5 between LPC and LPC-IPSCS were selected, resulting in the identification of 3065 probe sets that are most dissimilarly expressed between these two cell types. Expression pattern of these genes are shown as heat map for LPCs, LPC-iPSCs, and ESCs. Hierarchical clustering with Euclidean distance and average linkage was performed using all available probe sets (12088) and are shown as dendrogram. The difference in gene expression of LPC / ESCs and non-LPC / ESCs is shown as boxplots which give pairwise Euclidean distances of gene expression profiles (12088 probe sets) between the groups. Hierarchical clustering

with Euclidean distance and average linkage was performed for LPC, non-LPC, Ute and UT_dep samples using a set of 503 genes differentially expressed between LPC and non-LPC cells and between UT_en and UT_dep cells. Differentially expressed genes have been calculated by the shrinkage T-statistic[154] controlled for multiple testing by maintaining a FDR < 0.01[1, 155].

7.27 Lentiviral knock down of BAF complex components

To verify the effect of BAF complex components on the reprogramming in LPCs and non-LPCs, bacteria containing shRNAs against BAF155 and Brg1 were picked from pSM2 shRNA library from Open Biosystems and cultivated at 32°C overnight. The shRNAs were then cloned in the SFFLV lentiviral backbone and the lentivirus was produced as described above. The knock down of these shRNAs was validated by transducing Cgr8 ESCs. A scramble shRNA virus was used as a control. Knock down of Brg1 and Baf155 was measured via qPCR using QuantiTect primer assays (Qiagen). For iPSCs formation, 2000 freshly isolated LPCs and non-LPCs were seeded, followed by shRNA virus infection on day 1. The following day StemCCA reprogramming virus was added for additional 24 hours. The next day the medium was changed to ES cell medium and mitomycin C inactivated feeder cells were added. After 10 days, cultures were fixed and stained for alkaline phosphatase. Colonies were counted under a Leica microscope according to ES cell morphology and AP positive staining signal.

7.28 Statistical analysis

According to the results of the univariate test, continuous variables were expressed as means ± SD. The data was analyzed using the software Graphpad Prism 5. The p values were calculated using two-tailed unpaired Student’s t-test and a p value ≤ 0.05 was considered statistically significant.

8 Results

8.1 Isolation and characterization of liver progenitor cells

Liver progenitor cells were isolated from both foetal and adult liver based on previously described surface markers (See Materials and Methods 7.8) from Oct3/4-eGFP reporter mice. The cells of the Oct3/4-eGFP reporter mice provide an advantage to identify fully reprogrammed iPSC colonies (See Materials and Methods section 7.7). In differentiated cells, the Oct3/4 promoter is methylated and as a result the Oct3/4 protein is not expressed. During the process of reprogramming the Oct3/4 gene promoter is demethylated thus expressing Oct3/4 protein. In the above mentioned mouse model, this demethylation of the Oct3/4 promoter locus helps the transcriptional machinery to access it which in turn switch on the expression of eGFP reporter. Thus, the eGFP expression in the developing iPSC colonies substantiate the formation of fully developed iPSCs colonies expressing Oct3/4 [156].

For foetal liver, CD13+ and Dlk+ double positive cells were gated as liver progenitor cells and CD 13- and Dlk- cells were sorted as non-LPCs **(Figure 9A)**. It is known that the expression of Dlk goes down in the foetal liver after day E15.5 and is completely lost in adult liver. However, the expression of CD49f increases in adult liver [149]. Hence, for adult liver CD13+ and CD133+ double positive cells were gated and further analysed for the expression of CD49f **(Figure 9B)**. These CD13+, CD133+, CD49f+ triple positive cells were sorted as LPCs and triple negative cells as non-LPCs.

Based on the above sorting strategy, the LPCs contributed to approximately 12.7% of the whole liver cell population from foetal liver while approximately 79% of the marker negative subset of cells was sorted as non-LPCs. However, in adult murine liver the marker positive population declined and was found to be present less than 1% which was sorted as the progenitor cells. Marker negative population was found to be approximately 80% of the total population in adult murine liver.

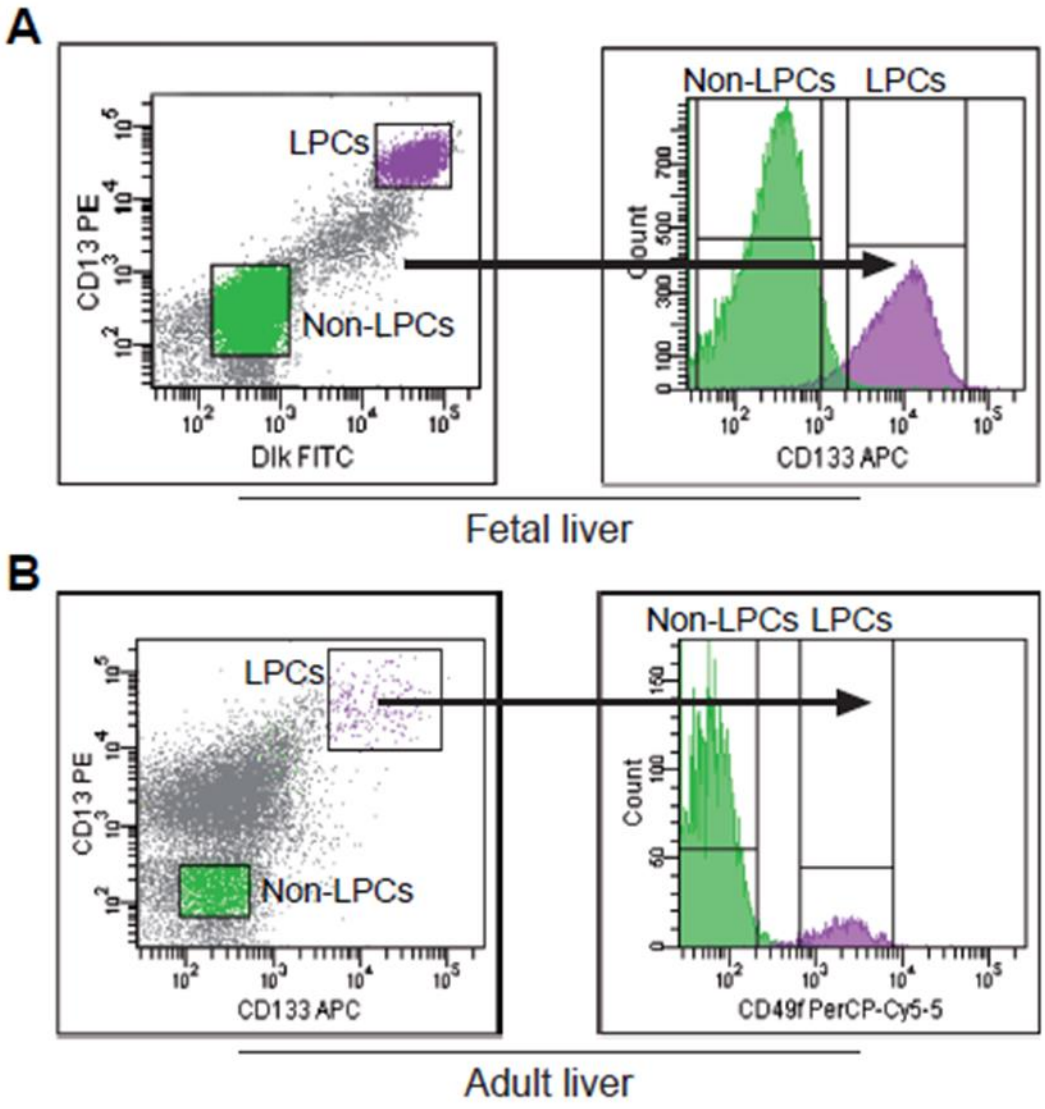


Figure 9 Schematic illustration of LPC isolation. (A) Representative FACS dot blots obtained after staining of dissociated foetal liver with anti-CD13 and anti-DLK antibodies (left panel) and anti-CD133 antibody (right panel). Squares mark the gates for sorting liver progenitor cells (LPCs, marker positive) and non-LPCs (marker-negative). (B) Representative FACS dot blots after staining of dissociated adult liver with anti-CD13 and anti-CD133 antibodies (left panel) and anti-CD49f antibody (right panel). Squares mark the gates for sorting non-LPCs (marker-negative) and LPCs (marker-positive).

8.2 Liver progenitor cells are bipotent

Recent data from our own lab and also various other labs confirmed the presence of an enriched population in the liver cells which gave rise to tumours of bilinear origin [149, 157]. These subset of cells when sorted as single cells showed clonal expansion capacity and formed colonies which expressed both the markers of cholangiocytic as well as hepatocyte lineage [157]. These cells were also known to self renew. Thus LPCs pose as an enriched subset for CD13(+)CD49f(+)CD133(+) cells, which can prove efficient to be an ultimate cell source for reprogramming as it shares some properties of adult stem cells such as self renewal [149].

To assess the clonal growth and efficiency of these cells, LPCs were sorted as single cells based on the surface marker profile as mentioned above in 96 well plates. Approximately, 25% of the singe cells gave rise to colonies after one week of culture. These colonies when stained for specific markers showed a mixed lineage differentiation into both cholangiocytes and hepatocytes **(Fig 10A)**. The experiment was performed in triplicates by isolating the cells from three different embryos. The bilineal differentiation of these cells was revealed by immunofluorescence staining for cytokeratin 19, a marker for cholangiocytes and albumin; a hepatocyte marker **(Figure 10B)**.

Together, these data support the presence of a progenitor/stem cell population in the liver which can exhibit some properties of the adult stem cells such as self renewal and differentiation into more matured cells.

A

		Colony number per 96 well plate	Colony formation [%]	Mean values [%]
Embryo 1	Plate 1	22	22,91	21,87
	Plate 2	20	20,83	
Embryo 2	Plate 1	21	21,88	25,00
	Plate 2	24	25,00	
	Plate 3	27	28,13	
Embryo 3	Plate 1	18	18,75	17,36
	Plate 2	18	18,75	
	Plate 3	14	14,58	

B

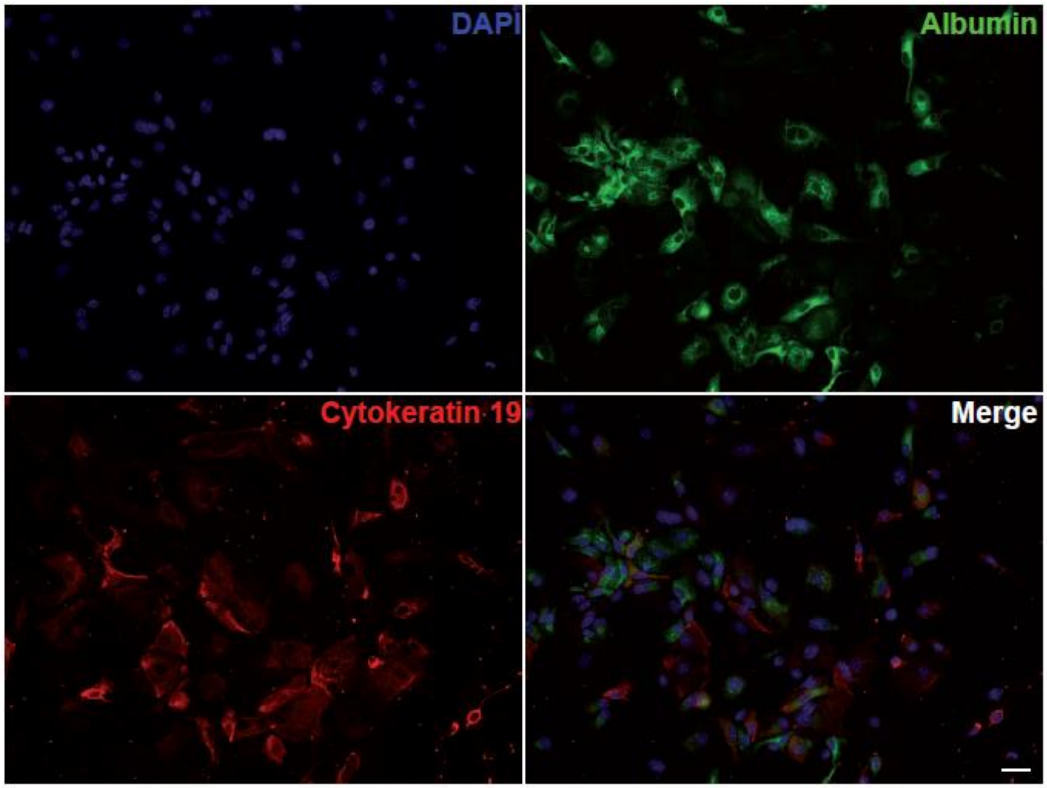


Figure 10 Bilineal differentiation of the LPCs into more matured cells. A) Table depicting the average percentage of the colonies formed by the single LPCs sorted on 96 well plates which gave rise to the mixed differentiation. B) Immunofluorescence staining for the hepatocytic marker albumin (green) and the cholangiocytic marker K19 (red) of LPCs of the indicated bilineal differentiation of the arising colonies into both cholangiocytes as well as hepatocytes (merge). Scale bars, 100 µm.

8.3 Transcriptome analysis of liver progenitor cells

Previously, various groups had isolated liver progenitor cells using different methods and markers. To verify the authenticity of the sorted LPCs as an enriched cell population present among the whole liver, a whole transcriptome analysis was performed on the samples. As a control, DCC (3,5-diethoxycarbonyl-1,4-dihydrocollidine or diethyl1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate) untreated (UT) enriched LPCs and UT depleted LPCs were used from previously described studies [158, 159]. 503 differentially regulated genes were analysed and compared in between our samples (LPCs and non-LPCs) and the previously described samples (UT enriched and UT depleted). After doing a hierarchical clustering analysis we observed that the gene expression pattern of sorted LPCs was identical to the gene expression pattern of enriched subset of cells from the liver in previously published data. On the other hand, the transcriptome profile of non-LPCs proved to be different from LPCs and previously described progenitor compartment from liver **(Figure 11)**. This correlation between resemblances of gene expression pattern in LPCs to the already published data sets gave a positive signal to the fact that the isolated LPCs are an enriched population in the liver cells.

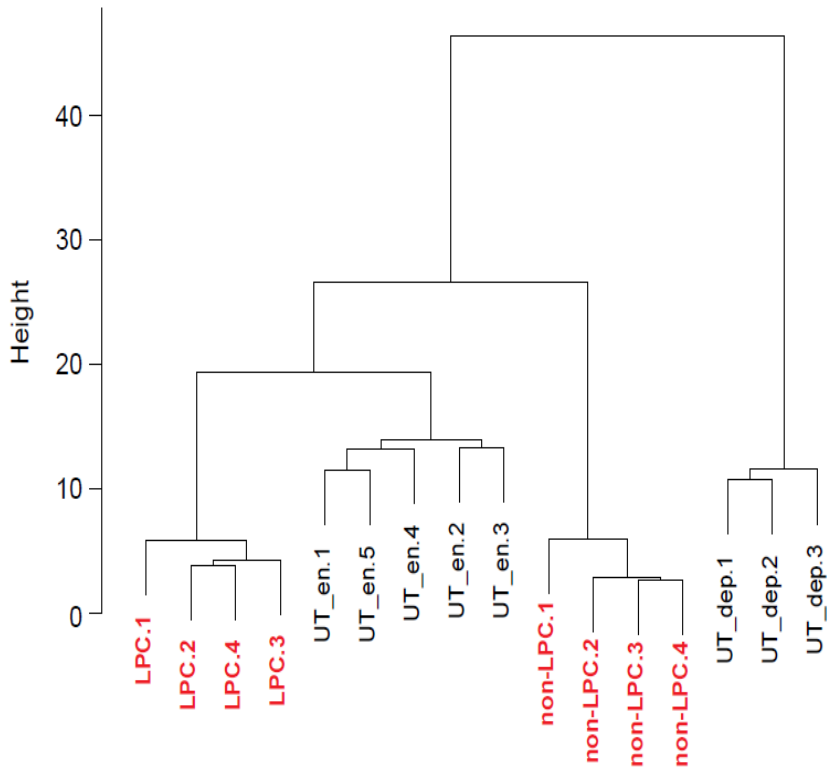


Figure 11 Hierarchical cluster analyses of LPCs, non-LPC cells. Comparison of LPCs, non-LPCs, UT enriched and UT depleted cells [158] based on a set of 503 genes differentially expressed between LPC and non-LPC samples and between UT_en and UT_dep samples. Own samples are shown in red.

8.4 Differentiation stage determines potential of murine liver cells for reprogramming into induced pluripotent stem cells.

8.4.1 Reprogramming with OKSM

To check whether the differentiation stage of the cell affects its reprogramming potential, LPCs and non-LPCs from both foetal and adult Oct-eGFP reporter mouse livers were FACS sorted and reprogrammed. Two types of reprogramming strategies were used- four factor approach using polycistronic construct encoding Oct3/4, Sox-2, Klf4 and c-Myc and three factor approach consisting of vector encoding Oct3/4, Sox2 and Klf4 **(Figure 12A)**. After the transduction with all four reprogramming factors, the LPCs started to reprogram as early as on day 3 giving rise to small clusters of cells which were Oct3/4-eGFP positive indicating the reactivation of the Oct3/4 locus in these cell **(Figure 12B)**. Ten days post transduction, the LPCs from both foetal as well as adult mouse livers showed increased iPSCs colony formation strongly characterized by the presence of alkaline phosphatase (AP) staining while the non-LPCs from both foetal as well as adult mouse livers only showed sporadic iPSCs formation (foetal LPCs 226±23 vs. non-LPCs 3±1) **(Figure 12C)**. Virtually all AP-positive colonies showed reactivation of the Oct3/4-locus as assessed by eGFP-fluorescence **(Figure 12D)**.

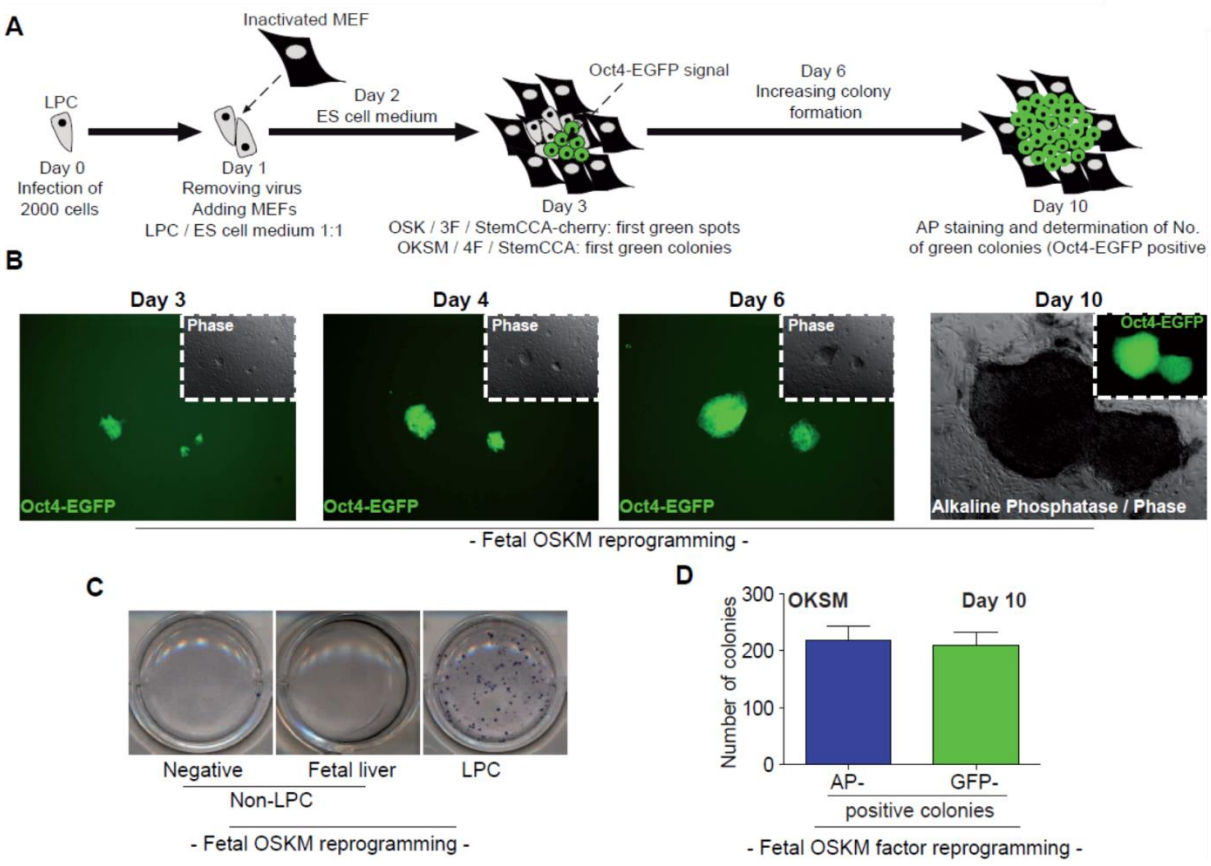


Figure 12 Cellular reprogramming of progenitor and differentiated liver cells using four reprogramming factors. (A) Schematic outline of the experimental course of cellular reprogramming of LPCs into iPSCs. 2,000 liver cells were sorted and infected with OSK or OSKM expressing lentiviruses. Labelling below and above the horizontal axis indicates culture conditions. (B) Time course of reprogramming of LPCs infected by a lentivirus expressing OSKM. Representative images showing eGFP activation of endogenous Oct3/4 reporter; morphological changes during reprogramming (phase contrast). Pictures at day 10 show the GFP signal and the alkaline phosphatase staining of a single (identical) iPSCs colony. (C) Representative AP-staining of iPSCs colonies of 4F reprogrammed LPCs, non-LPCs (marker-negative cells) and non-purified cells from mouse foetal liver at day 10 after viral infection. (D) Number of colonies of OSKM-infected foetal liver progenitor cells showing Oct3/4-EGFP expression or AP-staining on day 10.

8.4.2 Reprogramming with OKS

It has been already shown in many studies that even the use of three extrinsic reprogramming factors is enough to induce reprogramming in some cell types. The higher reprogramming efficiencies in the case of LPCs after 4F transduction prompted the use of 3F constructs. After transduction with the 3 reprogramming factors namely Oct3/4, Sox2 and Klf4, the iPSCs colony numbers in LPCs strongly exceeded than those in non-LPCs indicating a similar phenotype as observed after reprogramming by 4F. The colonies in the LPCs arose earlier than those in the non-LPCs **(Figure 13 A-C)**. When compared with the 4F reprogramming, the colony numbers were slightly reduced in the case of 3F reprogramming (foetal: 122±5.1 vs. 1.75±0.41; adult: 134±11 vs. 8±1) nevertheless, all the AP positive colonies showed a reactivated Oct3/4-EGFP locus shown by EGFP fluorescence **(Figure 13D)**.

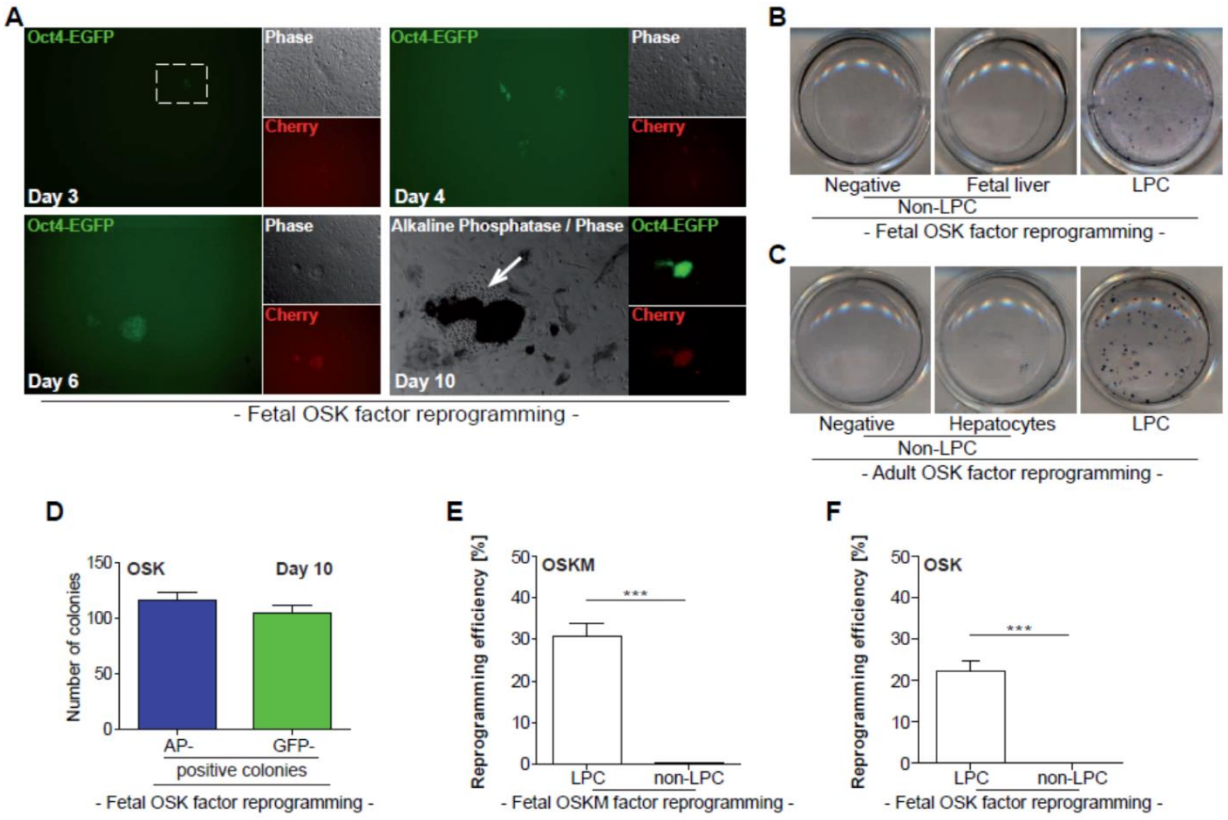


Figure 13 Cellular reprogramming of progenitor and differentiated liver cells using three reprogramming factors and calculated reprogramming efficiencies for OSK and OSKM-mediated reprogramming. (A) Time course of OSK (3F) reprogramming to the reprogrammed iPSCs state (days 3, 4, 6, 10). Endogenous Oct3/4-eGFP signal (dashed square, green), mCherry expression encoded by the polycistronic lentivirus (red), morphological changes during reprogramming (phase contrast). (B) Representative AP-stainings of a 12-well of 3F reprogrammed foetal LPCs and non-LPCs (C) Representative AP-stainings of a 12-well of OSK reprogrammed adult LPCs and non-LPCs (D) Number of colonies of OSK-infected foetal liver progenitor cells showing Oct3/4-EGFP expression and alkaline-phosphatase staining on day 10. (E) The histogram shows the infection efficiency-corrected reprogramming efficiencies by 4 factors of LPCs and non-LPCs from foetal liver. (F) The histogram shows the infection efficiency-corrected reprogramming efficiencies by 3 factors of LPCs and non-LPCs from foetal liver ***, $p < 0.0001$

8.5 LPCs and non-LPCs virtually show similar transduction rates.

8.5.1 Determining transduction rates in different liver cell compartments

The transduction rates was calculated to be similar in both PCs and non-LPCs. Transduction-corrected reprogramming efficiencies of LPCs were up to 32% for 4-factor experiments (OSKM) and 22% for 3-factor experiments (OSK) **(Figure 13E and F).** These reprogramming efficiencies were significantly higher when being compared to non-LPCs (1.2% for OSKM-infected cells; 0.08% for OSK-infected cells; $p<0.0001$) (**Figure 13E and F)** Of note, the reprogramming rate of LPCs exceeds the highest so far reported reprogramming efficiencies achieved in other studies [4, 134, 139, 160-163] and non purified cells revealed efficiencies in line with previous reports for somatic cell populations such as MEFs. The reprogramming efficiencies of the LPCs after 4F reprogramming were estimated to be around 30% whereas the reprogramming efficiencies of the non-LPCs were 0.34% **(Table 1)**. So a natural question would be to ask whether these differences are arising due to the differential transduction rates of LPCs or non-LPCs with the STEMCCA virus.

Table 1: The table below presents a detailed view of reprogramming efficiencies and their corresponding transduction corrected reprogramming efficiencies using 4F (OKSM) represented by the number of AP as well as GFP positive colonies in LPCs and non-LPCs.

No. of reprogramming factors	Respective cell type	No. of AP-positive colonies	No. of GFP-positive colonies	Transduction efficiency	Transduction corrected reprogramming efficiency (%)	
					No. of AP-positive colonies	No. of GFP-positive colonies
OKSM	LPC (marker positive cells)	226 ± 23	217 ± 23	38 ± 8	30,3 ± 3,27	30,04 ± 3,28
OKSM	non-LPC (unsorted fetal liver)	3 ± 1	3 ± 1	34 ± 7	0,34 ± 0,13	0,34 ± 0,13

To directly calculate the transduction corrected reprogramming efficiency of different liver cell populations showing equal infection rates in both the LPCs and non-LPcs, a series of experiments were performed. Firstly, we used STEMCCA-3F-Cherry

polycistronic lentiviral construct to transduce both LPCs and non-LPCs. Since STEMCCA-3F-Cherry vector has a mCherry reporter, red fluorescence upon the expression of the cassette enables to measure the transduction efficiencies based on positive red cells. When transduction efficiency in LPCs was normalized to 1 it was observed that there is no significant difference of transduction efficiency between LPCs and non-LPCs **(Figure 14A).** Thus, the infection rates of both LPCs and non-LPCs were virtually similar after the infection.

Secondly quantitative PCR primers specific for STEMCCA cassette flanking the 2A peptides were designed to measure its expression at mRNA level. No significant difference was observed between the expression of STEMCCA cassette in LPCs and non-LPCs **(Figure 14B).** Finally to exclude the possibility that those STEMCCA viral particles can show different integration in genomic DNA of LPCs and non-LPCs we performed a quantitative PCR on genomic DNA using specific primers for STEMCCA. It was found that STEMCCA shows a very similar integration and there is no significant difference in copy numbers of STEMCCA between and LPCs and non-LPCs **(Figure 14C)**.

To this end we can conclude that there are no relevant differences in transduction rates between LPCs and non-LPCs and the determined rates can be used in further calculations of a reprogramming efficiency.

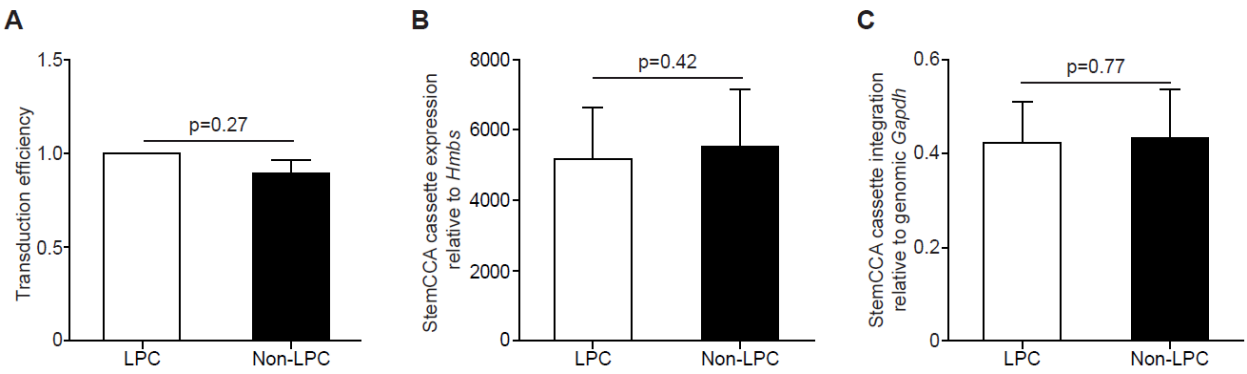


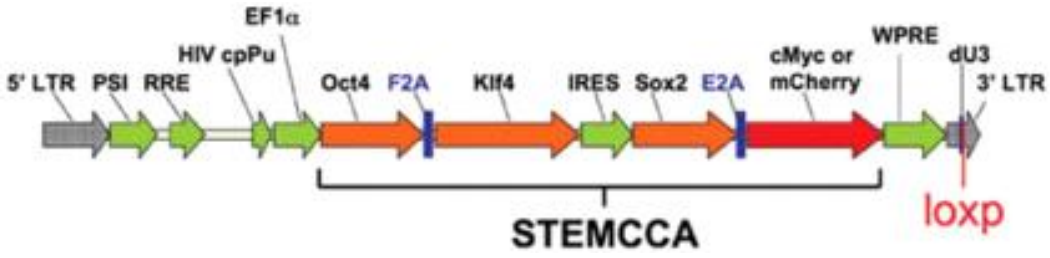
Figure 14 LPCs and non-LPCs have no significant differences in transduction efficiencies, expression and integration pattern using StemCCA-lentiviral particles. (A) Tranduction rates in both LPCs and non-LPCs depicted by number of positive cells in LPCs set to 1 and transduction rates in non-LPCs are depicted as fold induction. (B-C) Expression of the StemCCA cassette after the transduction of LPCs and non-LPCs on either mRNA (B) or genomic DNA (C). Primers specifically targeting the StemCCA cassette were used to analyze either expression at mRNA level (B) or to measure integration (C) of the StemCCA cassette expression in genome of LPCs and non-LPCs.

8.6 LPC-derived iPSCs show hallmarks of embryonic stem cells

8.6.1 Expression of pluripotency markers in LPC-iPSCs

Embryonic stem cells are characterized based on their surface markers as well as by the expression of certain transcription factors, which maintain their pluripotent state. ESCs usually express a surface marker called Stage Specific Embryonic antigen 1 (SSEA1) in mouse and SSEA4 in humans [164, 165]. Induced pluripotent stem cells derived from MEFs or any other cell type are also known to express this surface marker as iPSCs are similar to the ESCs. As a result, SSEA1 surface marker expression is likely to be a confirmation for the formation of a true iPS cell colony from differentiated cells [166]. To verify the authenticity of the LPC derived iPSCs, single colonies from the plate were picked on day 10 and cultured further in ES cell conditions to expand the colonies and to establish single cell lines. Interestingly, all the established iPSC clones from adult and foetal LPCs stained positive for SSEA1 surface marker proving their validity as iPSCs having embryonic stem cells characteristics **(Figure 15A-B)**.

Another pluripotency marker tested to authenticate the LPC derived iPSCs clones was the transcription factor Nanog. Nanog is a homeobox protein which in concert with Oct3/4 and Sox2 helps in self-renewal of ESCs thereby maintaining their pluripotent nature [35]. Since, Oct3/4 and Sox2 were already a part of reprogramming cocktail, the activation of endogenous Nanog expression points to the complete reprogramming of the LPCs. All the established clones from both adult and foetal LPCs strongly expressed Nanog **(Figure 15A-B)**. The modified 3 factor (3F) vector containing the reporter mCherry instead of cMyc expresses RFP in the cells under the constitutive EF1α promoter. However, the fully formed iPSCs colonies are known to silence the proviral transgene in subsequent passages. After the silencing of the transgene, the cells however maintain their pluripotency status [167]. The activation of the Oct-EGFP locus in our Oct-EGFP reporter mice together with the strong silencing of the cherry-v-transgene also prove the complete reprogramming process in the LPCs **(Figure 15A-B)**.



Schematic representation of the STEMCCA construct

An mRNA expression analysis of various pluripotency genes like Oct3/4, Sox2, Klf4, c-Myc, Nanog, Rex1 and c-Kit showed similar levels of the pluripotency genes when compared with the ESCs **(Figure 15 C-D)**. However, the expression of Nanog significantly increased compared to the ESCs suggesting a strong reactivation of the endogenously expressed pluripotency loci **(Figure 15D).**

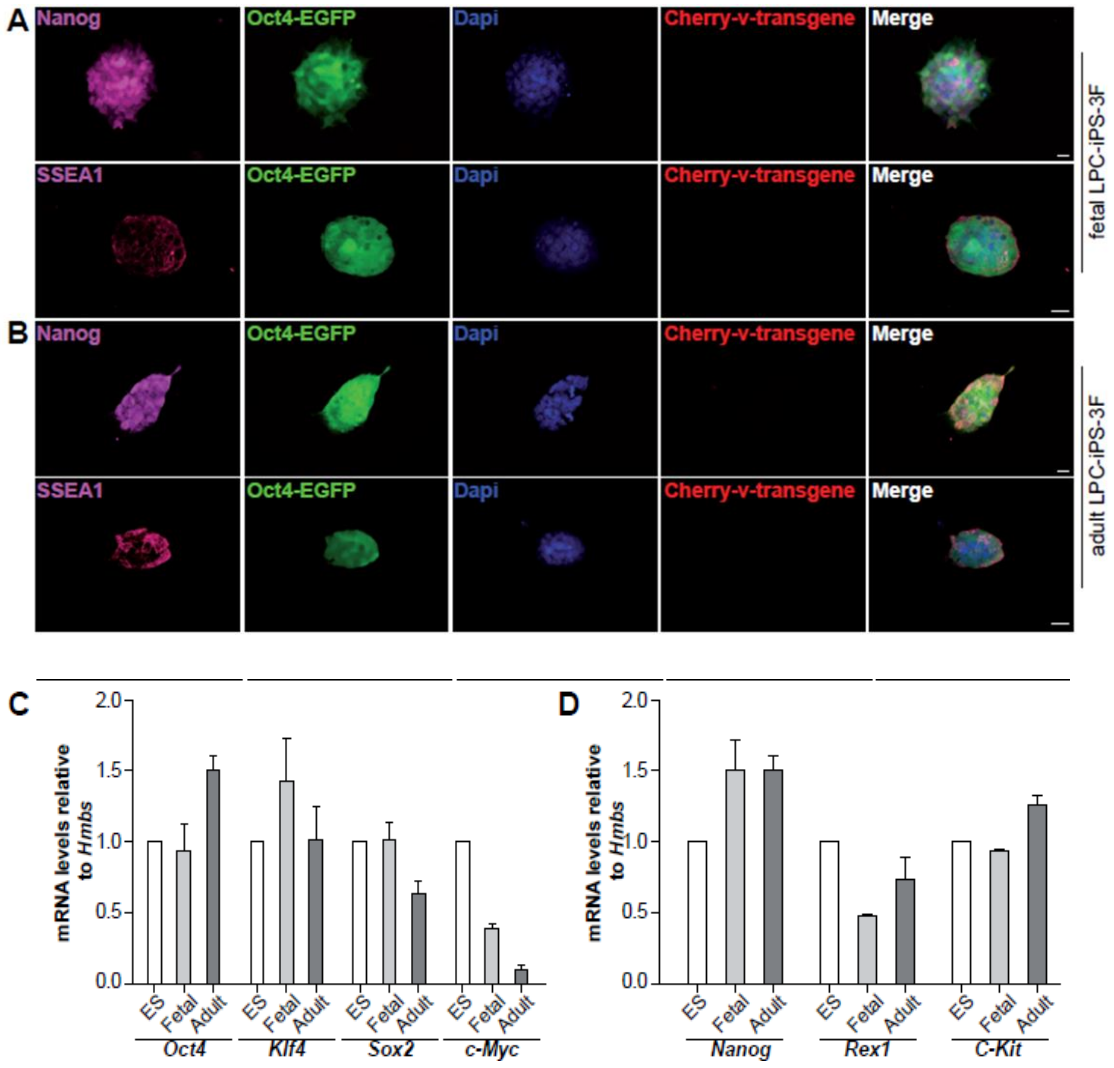


Figure 15 Expression of pluripotency markers in iPSCs derived from LPCs. IPSCs were generated from either foetal or adult LPCs using an OSK-encoding lentivirus (LPC-iPSCs-3F). IPSCS were analyzed by immunofluorescence and qPCR for the expression of pluripotency proteins/genes. (A-B) Immunofluorescence staining on foetal (A) and adult (B) LPC-derived iPSCs clones shows reactivation of Nanog (upper section, magenta) and SSEA1 (lower section, magenta). Oct3/4 reactivation is shown by reactivation of the EGFP reporter locus (green). The suppression of the viral transgene was indicated by mCherry expression construct (red). Scale bars: 10µM. (C-D) qPCR analysis of the mRNA expression of pluripotency-associated genes in ESCs and established foetal and adult LPC-iPSCs-3F clones. Expression of the respective marker in ESCs was set to 1. (C) Oct3/4, Klf4, Sox2 and c-Myc. (D) Nanog, Rex1 and c-Kit. Data are representative for all established LPC-iPSCs clones.

8.6.2 LPC-derived iPSCs can differentiate into all germ layers

ESCs can differentiate into all the three germ layers. This feature of the ESCs is also shared by the induced pluripotent stem cells being similar to the ESCs in their caricature. To test the differentiation potential of the LPC-iPSCs from both adult and foetal *in vitro*, embryoid body (EB) formation assay was performed using hanging drop technique for the cells to differentiate in differentiation medium. The Oct3/4-EGFP reporter expression made sure that the EBs were developed from true adult-LPC-iPSCs and foetal-LPC-iPSCs **(Figure 16A and Figure 17A)**.

The differentiation of the EBs into the mesodermal lineage was marked by the expression of a-actinin. a-actinin is an actin binding protein and is used as marker for the cardiac muscle formation and hence can be used as a marker for the mesodermal lineage [168]. Fluroscence staining of the differentiated EBs with a-actinin showed the presence of clusters of cells which were positive for the staining suggesting the possible differentiation of the cells towards mesodermal origin **(Figure 16 B and Figure 17 B)**.

To check the differentiation of LPC-iPSC clones from both adult and foetal liver towards the neural lineage, the cells were stained for early neuronal precursor marker Nestin usually expressed during the early stages of the development of the nervous system [169]. Immunostaining of differentiating EBs clearly showed a presence of premature neuronal differentiation of the LPC-iPSCs indicating the successful differentiation of the LPC-iPSCs towards ectoderm lineage **(Figure 16C and Figure 17C)**. Another widely regarded neuronal marker ß-tubulin expression was tested in the adult-LPC-iPSCs and foetal-LPC-iPSCs derived EBs to check for the differentiation of these cells towards neuronal lineage [170]. Immunostaining of the differentiated EBs from adult-LPC-iPSCs showed filamentous staining from the cells indicating the differentiation of the LPC-iPSCs towards the ectodermal or neural lineage.

The differentiation of the EBs towards the endodermal origin was noted by the use of early liver precursor marker Alphafeto protein [171]. Both the adult-LPC-iPSCs and foetal-LPC-iPSCs showed the a strong presence of AFP expression on the protein

level when stained during the differentiation process suggesting the differentiation of these EBs towards endodermal lineage **(Figure 16D and Figure 17 D)**.

Thus, the above results point towards the successful differentiation of the LPC-iPSCs from both adult as well as foetal livers into all the 3 germ layers. The silencing of the Oct3/4- eGFP reporter in the cells also showed the differentiation process as the differentiated cells have methylated Oct3/4 gene promoter thereby shutting the expression of the eGFP in this case **(Figure 16 B,C, D and Figure 17 B,C,D).** This protein expression of the clones also correlated with the up regulation of specific early and late differentiation genes at mRNA levels.

The up regulation of Nestin and ß-3-tubulin mRNA levels was detected in both the adult LPC-iPSCs and the foetal LPC-iPSCs; an increase in the expression of Nestin and ß-3-tubulin was noted as the days of differentiation progressed pointing towards the successful differentiation of the LPC-iPSC clones towards ectodermal lineage **(Figure 18 A,B)**. However, as Nestin and ß-3-tubulin are expressed during the early days of the development, a gradual increase in Nestin and ß-3-tubulin mRNA levels are observed till day 8 **(Figure 18A, B)**. In addition to a-actinin, two other mesodermal markers were used to check the expression of the mesodermal differentiation of the cells, Nkx2.5 and Myh6. Nkx2.5 is a marker for early cardiac differentiation while Myh6 is well known marker of the late [172] . Similar to the expression levels of the neuronal markers, the expression of Nkx2.5 and Myh6 also increased significantly as the days of differentiation increased **(Figure 18 D,E)**. A gradual up regulation of AFP mRNA transcripts were observed as the differentiation proceeded indicating the differentiation of the clones towards endodermal lineage **(Figure 18 C)**.

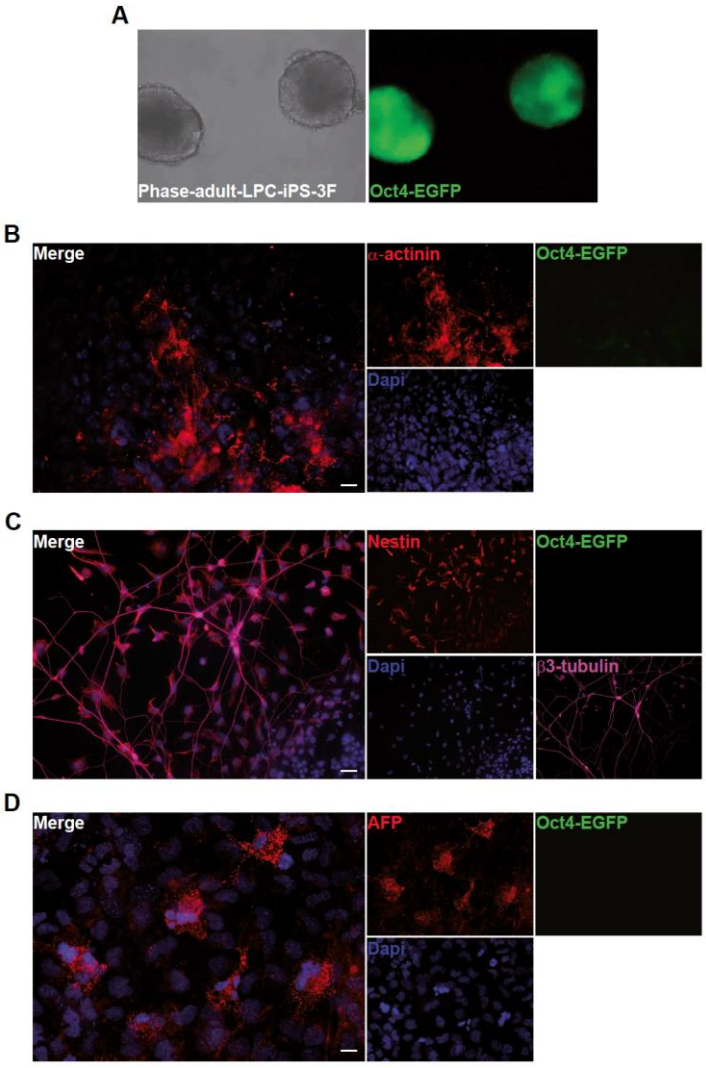


Figure 16 Immunofluroscence staining indicating the differentiation of the adult-LPC-iPSC into the three germ layers. (A) phase contrast image of the Ebs formed from the adult-LPC.iPSCs (left). Ebs expressing eGFP due to the presence of the active reporter driven by the Oct3/4 promotor indicating the pluripotent state of the Ebs before differentiation (right). (B) Immunofluroscence staining of the differentiating Ebs showing the expression of the mesodermal marker a-actinin (red) and the cells are stained with Dapi (blue). (C) The differentiating Ebs were capable to express the neuronal markers Nestin (red) and ß-3-tubulin (pink). Nuclei are stained with Dapi (blue). (D) Endodermal differentiation of the cells is showed by the presence of AFP (red). Nuclei are stained with Dapi (blue).

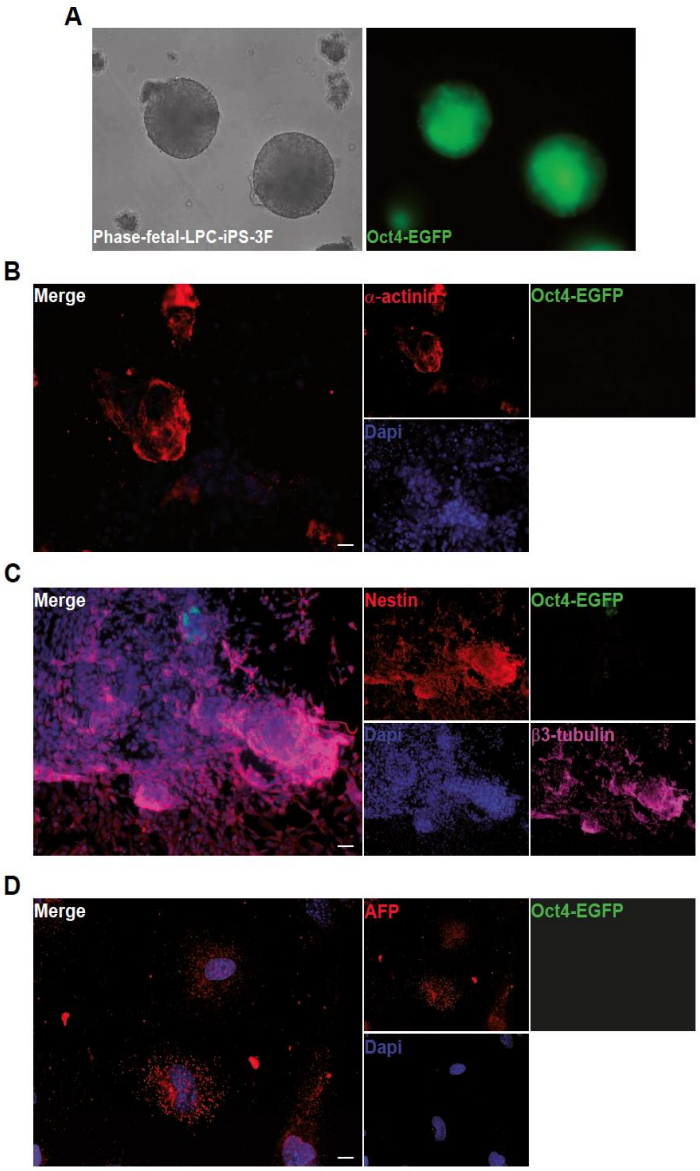


Figure 17 Immunofluroscence staining indicating the differentiation of the adult-LPC-iPSC into the three germ layers. (A) phase contrast image of the Ebs formed from the foetal-LPC.iPSCs (left). Ebs expressing eGFP due to the presence of the active reporter driven by the Oct3/4 promotor indicating the pluripotent state of the Ebs before differentiation (right). (B) Immunofluroscence staining of the differentiating Ebs showing the expression of the mesodermal marker a-actinin (red) and the cells are stained with Dapi (blue). (C) The differentiating Ebs were capable to express the neuronal markers Nestin (red) and ß-3-tubulin (pink). Nuclei are stained with Dapi (blue). (D) Endodermal differentiation of the cells is showed by the presence of AFP (red). Nuclei are stained with Dapi (blue).

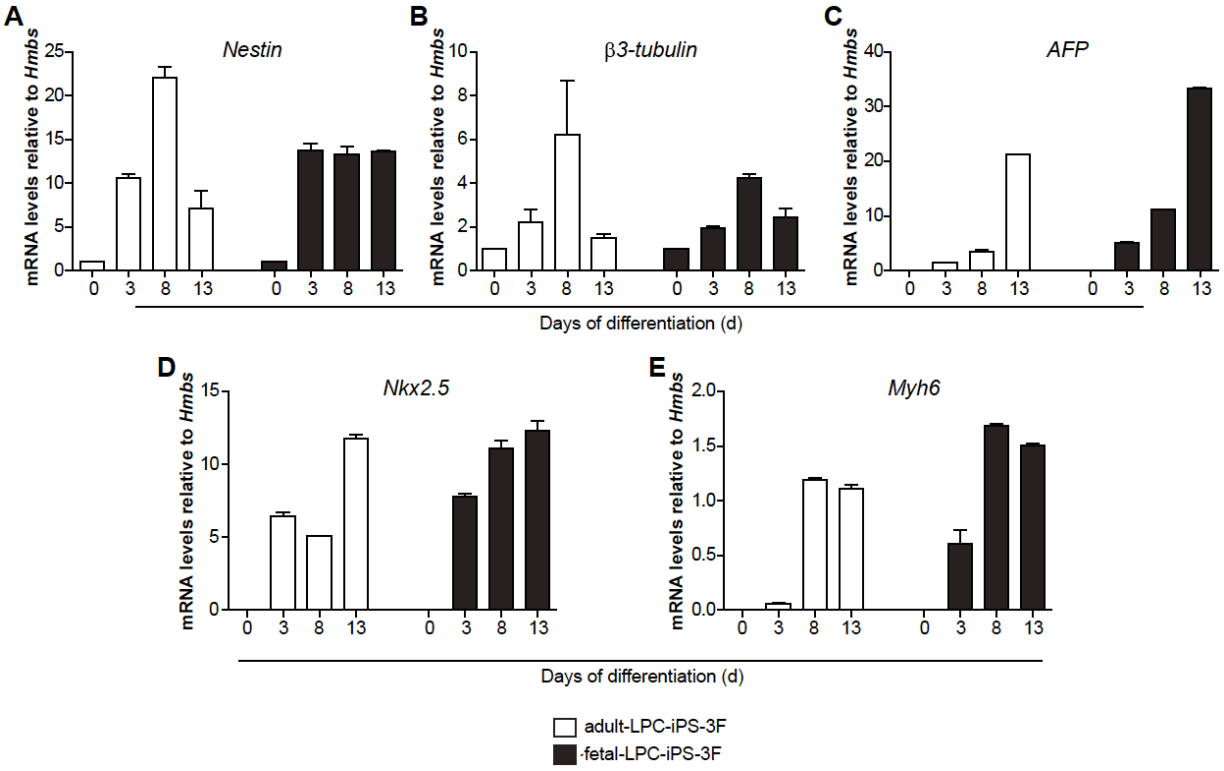


Figure 18 mRNA levels indicating the differentiation of the adult-LPC-iPSC and foetal-LPC.iPSCs into the three germ layers. (A, B) mRNA levels of neuronal markers Nestin and ß-3-tubulin (C) mRNA levels of endodermal marker AFP (D, E) mRNA levels of mesoderm markers Nkx 2.5 and Myh6. The mRNA levels of each genes were plotted against days of differentiation and were determined by qPCR. The white bars represent the gene levels in adult-LPC-iPSCs and the black bars represent the gene levels in foetal-LPC-iPSCs. All the gene levels were normalised to *Hmbs.*

8.6.3 Removal of the reprogramming cassette does not affect pluripotency

It is also of utmost importance to generate iPSCs without too many integrations of the provirus as the insertions lead by the virus can cause genetic malformations in the long run. Hence, there have been studies describing the formation of efficient iPSCs without the integration of the reprogramming cassette [119]. To assess whether the excision of STEMCCA cassette can lead still lead to stable iPS clones, we excised the cassette from the clones and established stable viral free cell lines.

The STEMCCA cassette is flanked at both ends with lox P sites thus allowing successful excision of the reprogramming cassette. Successfully established iPSCs clones were infected with a retroviral construct containing Cre-recombinase and puromycin resistance gene. Puromycin selection identified transduced clones. After several passages those clones were analyzed for the expression of pluripotency markers like Oct3/4 and SSEA 1. The presence of Oct3/4 regulated EGFP reporter enabled to mark the expression endogenous Oct3/4 in these cells. An intracellular antibody staining for Oct3/4 on the cells also proved the presence of Oct3/4 in the cells **(Figure 19A, B)**.

Along with endogenous Oct 3/4-eGFP expression, SSEA1 marker expression was also checked on transgene free adult and foetal LPC-iPSCs. Even after the excision of the transgene, the established clones expressed high levels of SSEA1 surface marker indicating the true iPSCs **(Figure 19A and B)**. These results clearly suggest that once the cells are fully reprogrammed they don't need the exogenous expression of reprogramming factors to maintain the state of pluripotency.

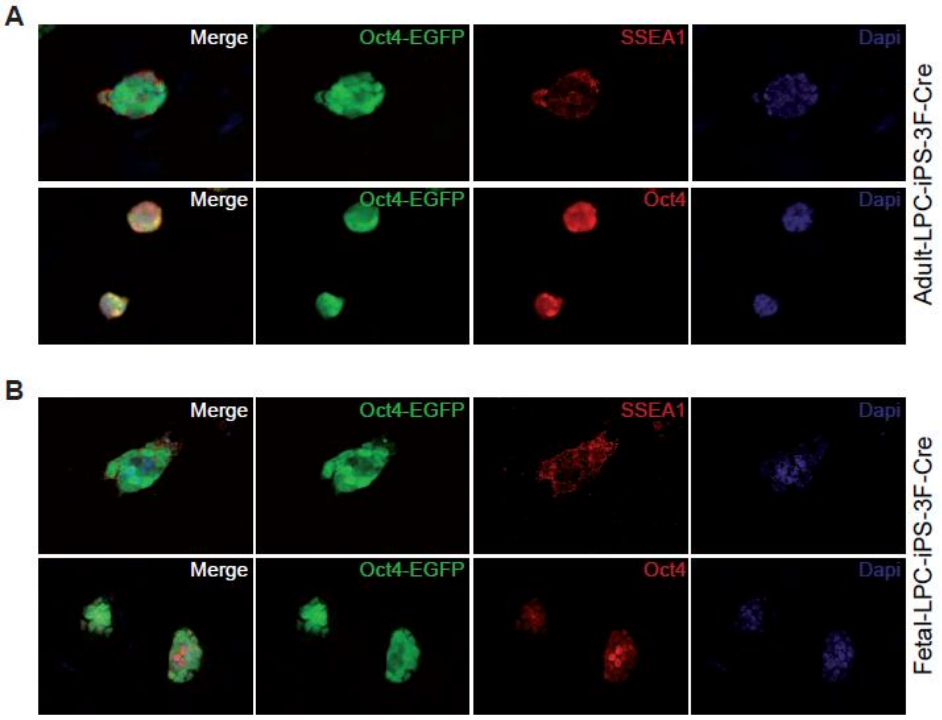


Figure 19 Removal of the floxed STEMCCA-3F cassette in foetal or adult LPC-derived 3F-iPSCs does not affect state of pluripotency. (A) foetal LPC-derived iPSCs and (B) adult LPC-derived iPSCs were infected with a retroviral construct expressing cre-recombinase and selected for a week on the a puromycin resistance. Following the selection, the cells were stained for pluripotency markers (Oct3/4, SSEA1) and also analyzed for the expression of endogenous OCT-4 GFP expression.

8.6.4 LPC-derived iPSCs efficiently form teratoma

The hallmark of ESCs is to form teratomas when injected in a immunosuppressant mice. Since, the generation of iPSCs *in vitro* could lead to genetic manipulations due to viral integrations, the formation of teratoma is considered to be an important pluripotency assay *in vitro*. Keeping this in mind, the 3F- LPC derived iPSC clones were counted on the basis of their activated Oct3/4-EGFP reporter and around 1 million cells were subcutaneously injected into immunocompromised II2-RG-/- mice. Four weeks later, mice with teratomas were killed and the tumors were sectioned and stained for H and E to identify all the three germ layers. The 3F-LPC derived iPSCs showed the potency to differentiate into all the three germ layers and this was reflected by the formation of neural rosettes which are derived from ectoderm, glandular regions derived from endoderm and cartilages derived from mesoderm **(Figure 20 A)**.

8.6.5 LPC-derived iPSCs contribute to the germ line

The most stringent pluripotency criteria for the iPSCs are successful germline transmission via blastocyst injection. The 3F-LPC derived iPSCs was injected into the blastocysts and then was transferred to pseudo-pregnant mice. On day 13.5, the mice were sacrificed and male embryos were analyzed for Oct3/4-EGFP reporter expression. 6 out of 8 male chimeras showed successful germline transmission which was reported by the EGFP expression due to the reactivation of the Oct3/4 reporter **(Figure 20 B)**.

8.6.6 Transcriptome of LPC-derived iPSCs resembles embryonic stem cells

To go one step further in analyzing the 3F LPC derived iPSCs clones, a whole transcriptome analysis was done on the 3F LPC derived iPSCs clones. The genes analysed during this profiling were compared to the already published ESC profile. The data confirmed the similarity of LPC derived iPSCs to the ESCs but are distinct

from the LPCs **(Figure 20 C)**. Scatter plot analysis of the global gene expression profiles obtained from the microarrays demonstrated a distribution pattern of gene expressions which were comparable to ESCs but completely different from LPCs. The black lines indicate 2-fold differences in gene expression levels between the paired cell populations **(Figure 20 D)**. Hierarchical clustering of various clones of LPC-iPSCs, LPC-iPSCs-Cre, ESCs, MEF-iPSCs, MEFs and LPCs based on their global gene expression patterns showed that the 3Factor-LPC-iPSCs clustered closer to the ESCs but distinctly away from LPCs **(Figure 20 E)**. Interestingly, the excision of the STEMCCA cassette from the LPC derived iPSCs mediated by Cre/lox positioned the LPC-iPSCs-Cre even closer to the genetic profile of the ESCs than LPC-iPSCs suggesting the efficient reprogramming of the LPCs into LPC-iPSCc **(Figure 20 C, E)**.

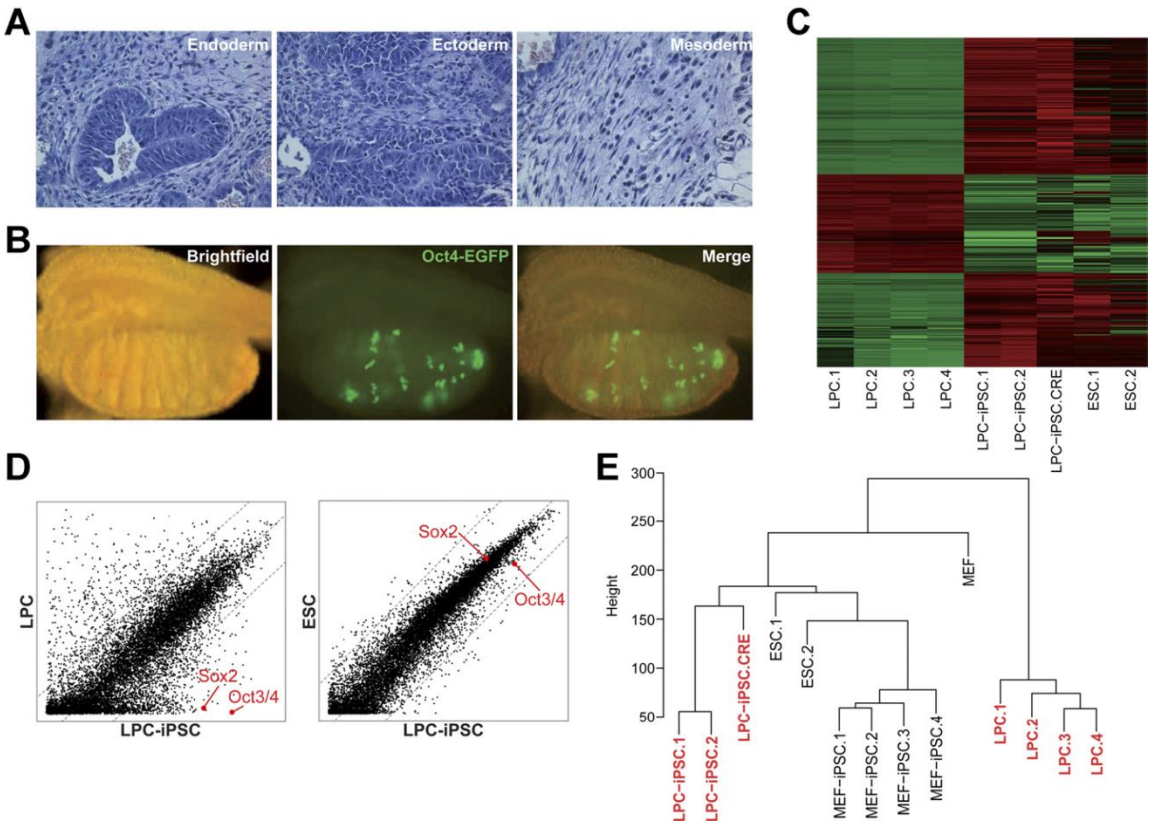


Figure 20 Teratoma formation and germline contribution of adult liver progenitor cell-derived iPSCs. (A) LPC-iPSCs form *in vivo* teratomas containing all 3 germ layers after the injection into NOD SCID mice. (B) LPCs-iPSCs were injected into blastocysts for chimera formation and then were for Oct3/4-EGFP-positive cells in the germline. (C) Expression pattern of genes that show a 5-fold difference in transcript levels between LPCs and foetal LPC-derived iPSCs including 1 cell line with Cre-mediated excision of viral transgene were chosen to plot a heat map. The same set of genes was analyzed in published profiles of ESCs (2 murine ES cell lines). (D) Scatter plots comparing global gene expression profiles between LPCs and LPC-derived iPSCs (left panel) and between ESCs and LPC-derived iPSCs (right panel). (E) Hierarchical clustering analysis of LPCs, LPC-derived iPSCs, MEFs, MEF-derived iPSCs, and ESCs based on genome-wide gene expression. Own samples are depicted in red.

8.6.7 LPC- and non-LPC-derived iPSCs do not show relevant differences in hepatic differentiation

Recent evidence suggested that the cell type of origin impacts the differentiation capacity of the respective iPSCs [137]. In this sense, iPSCs generated from pancreatic islets are more prone to differentiate to this lineage in the iPS cell stage compared to other iPSCs generated from a different tissue [136]. Thus, we hypothesised that maybe a similar but more restricted scenario could be the case in the liver namely that there are differences in hepatic differentiation in non-LPC, LPC-derived iPSCs. MEF-derived iPSCs served as a control of iPSCs for the mesodermal germ layer [139]. To check the epigenetic memory of the LPC derived iPSCs, the iPSCs were subjected to monolayer differentiation based on a cytokine approach mentioned above (See 7.21). After 20 days of induction of the LPC derived iPSCs from both adult and foetal liver with hepatic differentiation factors like dexamethasone, EGF and mHGF, hepatoblast and hepatocyte like morphology of the iPSCs after differentiation was observed. This led to the confirmation of the hypothesis that the LPC derived iPSCs tend to have an increase endodermal differentiation capacity **(Figure 21 A and B)**.

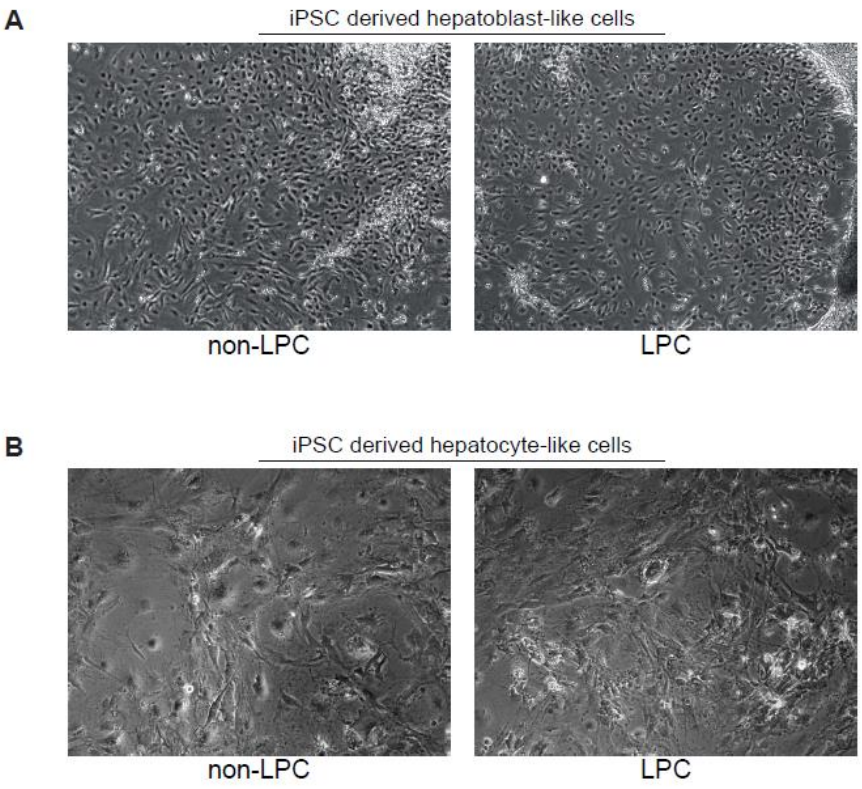


Figure 21 Morphology of the differentiated LPC derived iPSCs into hepatic cells. (A) Morphology of hepatoblast-like cells on day 20 of hepatic differentiation obtained from LPC-iPSCs and non-LPC-iPSCs, respectively.(B) Morphology of hepatocyte-like cells on day 20 of hepatic differentiation obtained from LPC-iPSCs and non-LPC-iPSCs, respectively.

In covenant with the previous results, LPC derived iPSCs showed a tendency to differentiate more towards the endodermal lineage in an hepatic monolayer *in vitro* assay [153]. MEF-iPSCs was used as a control. The LPC-iPSCs showed increased expression of endodermal and hepatic lineage markers such as Alphafeto protein (AFP) and albumin than the MEF-iPSCs **(Figure 22 A)**. Quantitative expression analysis of the mRNA levels for different endodermal markers such as brachyury, GATA4, AFP and albumin revealed nearly 10-50 fold higher expression of all the four markers in differentiated iPSCs from LPCs compared to the differentiated LPCs from MEFs **(Figure 22 C-D)**. The functionality of the differentiated iPSCs into the hepatocytes like progenitor cells was tested on the basis of CYP3A11 activity and the

amount of albumin secreted in the culture medium. Both the assays demonstrated a strong induction in the metabolic activity of the differentiated cells in terms of albumin and CYPA11 secretions in the culture medium **(Figure 22 B and E)** thereby proving that the LPC-iPSCs do posses epigenetic memory which eased their differentiation into the endodermal lineage easily. However, a more in depth analysis of these clones is required to put forth a conclusion suggesting the epigenetic memory preferences during differentiation.

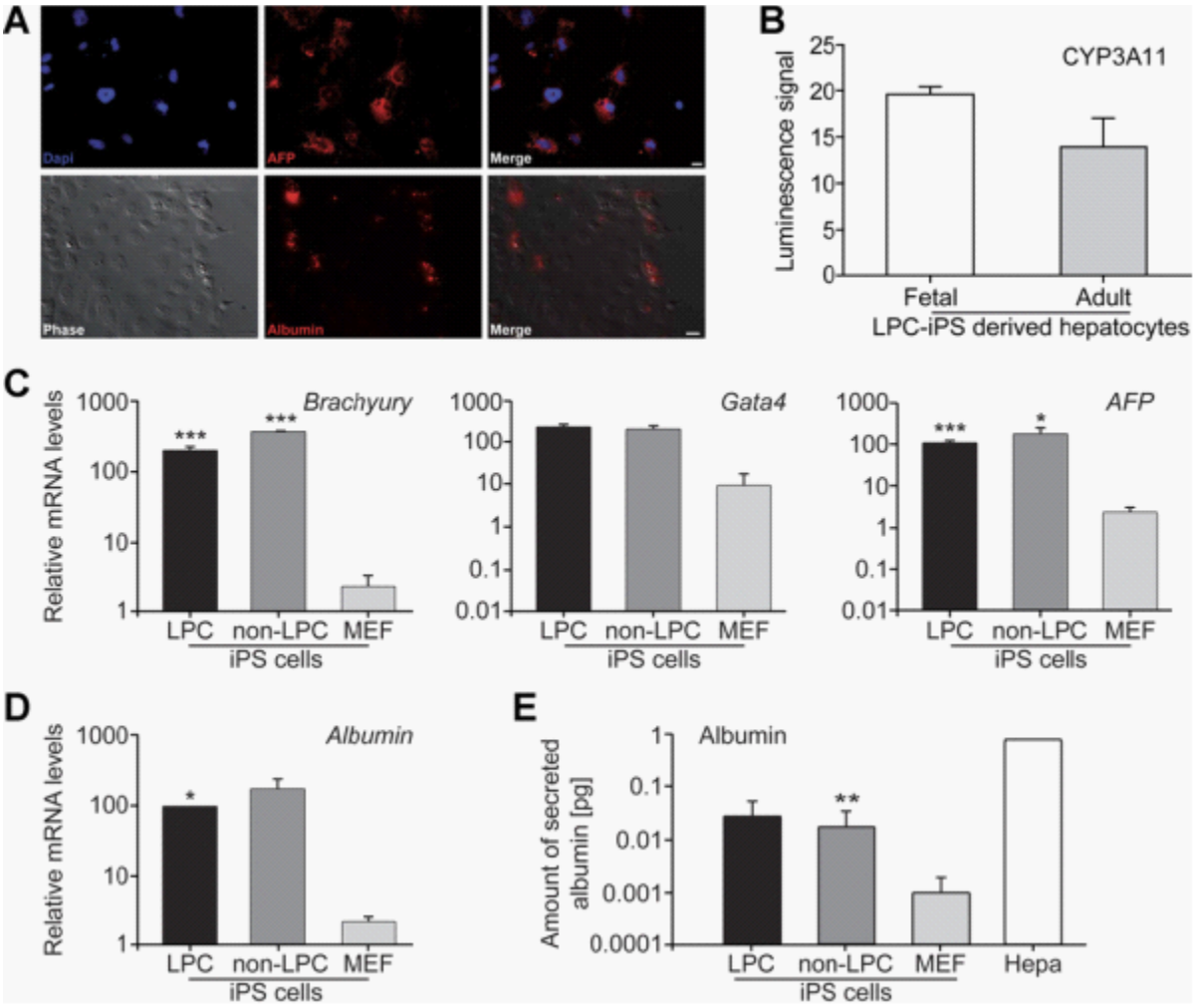


Figure 22 Hepatic Differentiation of the LPC-iPSCs and non-LPC-iPSCs. (A) On day 15, foetal-LPC iPSCs derived EBs were fixed and immunostained for endodermal derivates using

a alpha-fetoprotein (red) antibody as well as albumin antibody (red). Nuclei (DAPI, blue). Scale bar: 10μM. (B) The metabolic activity of CYP3A11 was measured in hepatocyte-like cells that were differentiated from either foetal or adult LPC-iPSCs-cre cells (day 20 after induction of differentiation). (C–E) Comparison of hepatic differentiation capacity of MEF-iPSCs, 3F-adult-LPC-iPSCs (LPC-iPSCs cells; passage 20) and 3Fhepatocyte-iPSCs (non-LPC-iPSCs cells; passage 16) using the same protocol as in panels A and B. (C) Induction of definitive endoderm (day 6 of hepatic differentiation) was evaluated by Brachury, Gata4, and AFP expression. mRNA levels are shown relative to undifferentiated ESCs as internal reference. (D–E) Maturation of hepatocyte-like cells was evaluated by albumin expression (day 20 of hepatic differentiation). (D) mRNA levels are shown relative to hepatoma cell lines 1–6 as internal reference. (E) Secretion of albumin into the culture supernatant after day 20 of hepatic differentiation is shown as the amount of albumin in 24 hours per 1000 cells (C–E; n _ 3).

8.7 Increased reprogramming efficiency of LPCs is independent of proliferation capacity

Recently, in a report from the Jaenisch lab it was hypothesized that proliferation rates influence the reprogramming efficiencies by inducing stochastic events [134]. To further analyze whether these differences in cell proliferation contribute to the increased reprogramming of the LPCs when compared to non-LPCs, proliferation rates were analyzed in these cells by two different approaches; bromodeoxyuridine labeling (BrDU) and propidium iodide (PI) staining. Both the dyes incorporate into the cellular DNA during cell proliferation and hence can be detected by FACS or by antibodies again BrDU. A slight increase in the proliferation of the LPCs compared to the non-LPCs was noticed **(Figure 23 A-B)**. After 6 hours of BrDU pulse on the freshly isolated and virus infected LPCs and non-LPCs, 64% cells were positive for BrDU in the LPCs compared to the 54% BrDU positive in the non-LPCs **(Figure 23 A)**. A similar result was observed in the PI assay where 37% of the LPCs were found in proliferation phase or S phase compared to the 29% of that of the non-LPCs **(Figure 23 B)**. When the iPSCs colony formation of these liver subpopulations were assessed in parallel to the cell cycle analysis, the LPCs showed much higher reprogramming efficiencies than the non-LPCs **(Figure 23 D)**.

A mathematical curve fitting analysis was done assuming that the reprogramming process would be delayed initially but over the time it would show similar reprogramming efficiencies in the non-LPCs too. The latency for the LPCs and the non-LPCs to reach a plateau of maximum reprogramming based on the observed data was calculated through this model. The latency for the non-LPCs to reprogram into iPSCs was calculated to be 10 folds diminished (69.77 days) compared to the latency for LPCs (6.21 days) **(Figure 23 C)**. Based on the previously published data, a double in the rate of proliferation resulted in a double reduction of the latencies in reprogramming of the cells [7, 134]. Keeping this in mind, the reprogramming kinetics of the LPCs was adjusted with their proliferation differences of 15%-20% than the non-LPCs **(Figure 23 A-B)** and the mathematical curve fitting analysis was again done. However, the increase in the proliferation rates of the LPCs by 15%-20% failed to

explain the 90% reduced latency of reprogramming in the LPCs compared to the non-LPCs **(Figure 23 E)**. The above observation led to the assumption that the proliferation rates may not be responsible for the high reprogramming efficiencies in the LPCs compared to the non-LPCs. To test this assumption, a theoretical decrease of proliferation by 50% in LPCs was included in the model of reprogramming kinetics and the curve was adjusted accordingly. Still, the decrease in the proliferation of the LPCs were not suitable to explain the 10 fold higher latency observed in non-LPCs compared to the LPCs **(Figure 23 E)**.

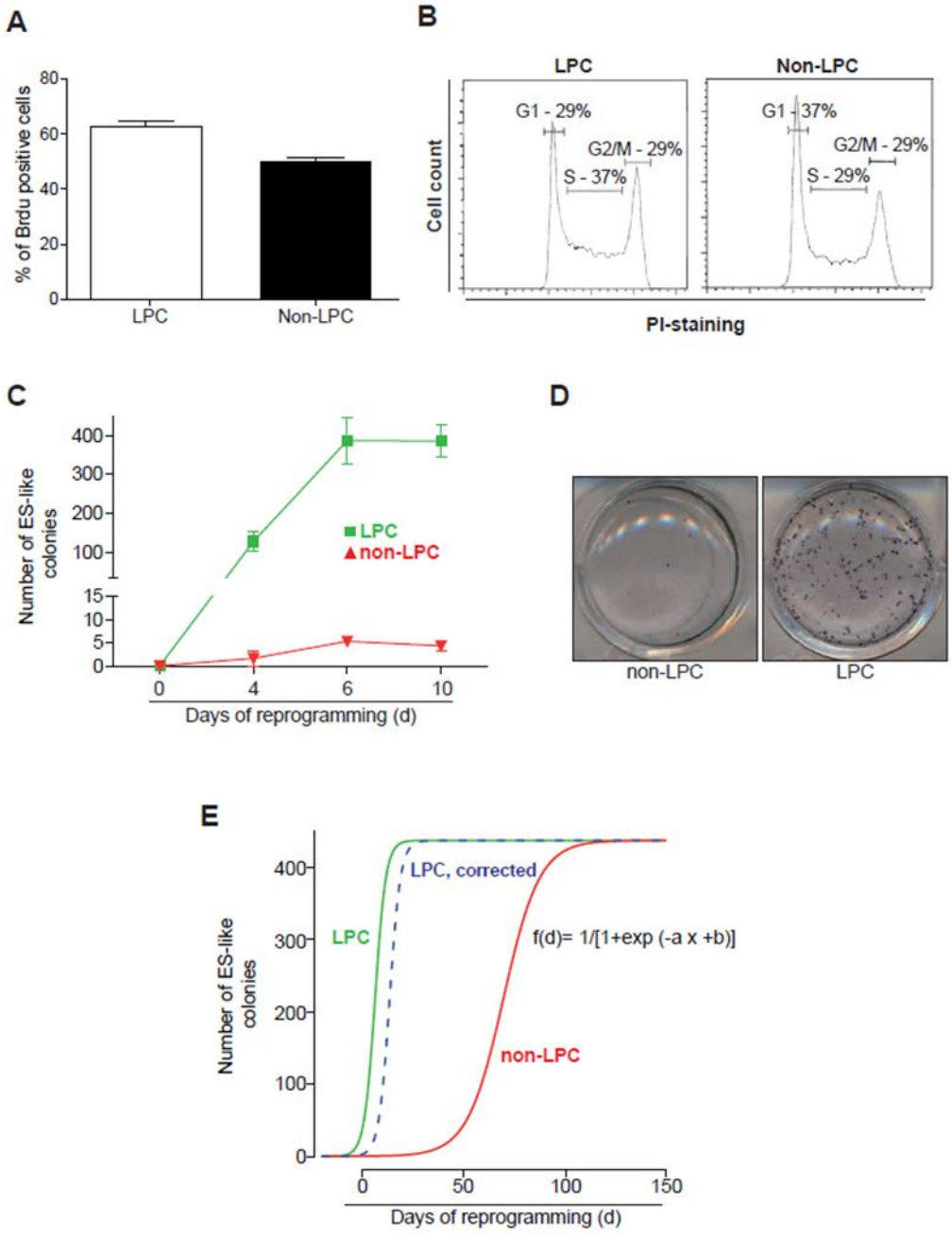


Figure 23 LPC and non-LPCs show similar proliferation capacities. (A) cell cycle analysis determined by Brdu staining on LPCs and non-LPCs showing the % of BrDU positive cells (B) cell cycle analysis determined by PI staining on LPCs and non-LPCs (C) Reprogramming kinetics of LPCs and non-LPCs after OKSM-infection (n=4). (D) Representative AP-staining at day 10 of one of the reprogramming kinetic experiments. (E) Mathematical curve fitting analysis of cellular reprogramming in LPCs and non-LPCs (n=5). All

experimental data were fitted (R version 2.11, function "nls") with sigmoidal functions of the form: f (d) = 1 / [1 + exp (-a x + b)]. Parameter values were: a = 0.372, b=2.311 (LPCs, green) and a=0.1116, b=7.7868 (non-LPCs, red). Inflection points were calculated to be at day 6.21 and day 69.77. Green line: reprogramming kinetics of LPCs. Blue line: predicted effect of a 50% decrease in cell proliferation on the delay in reprogramming of LPCs. Red line: calculated reprogramming kinetics of non-LPCs based on the measured data.

8.8 Increased reprogramming efficiency of LPCs is independent of endogenous expression of reprogramming factors

Recent reports in the past have established the endogenous expression of the some of the reprogramming factors in the adult stem cell population or the progenitor cell population [173, 174]. It has also been hypothesised that the endogenous expression of these factors can have a dual role in the adult stem cells or progenitor cells [175]; on one hand, they can help to maintain the stemness of the adult stem cells (ASCs) by driving the cell cycle progression making them closer to the pluripotent cells [175] while on the other hand, in presence of external reprogramming factors these factors can switch their role and aid in the process of reprogramming [73].

In order to gain insights whether this hypothesis holds true in the case of LPC reprogramming, LPCs were freshly isolated from both foetal (E14.5) and adult mice (6-8 months) by the above mentioned protocol and the endogenous expression of the reprogramming factors namely Oct3/4, Sox2, Klf4 and c-Myc was analysed on the mRNA levels. Expression levels of all the genes were normalized to a housekeeping gene mHmbs. A 3- 4 fold up regulation of Klf4 was detected in the LPCs compared to the non-LPCs in the foetal **(Figure 24 A)**. This expression was not unambiguous in the adult liver cells. Contrary to the expression in foetal LPCs, there was no significant expression of Klf4 in adult LPCs but the reprogramming efficiencies were similar in both foetal and adult LPCs **(Figure 24 A-B)**. The other factor, which showed significantly higher endogenous expression in both the foetal and adult LPCs, is the c-Myc. In adult liver progenitor cells, robust expression of c-Myc was observed in the LPCs compared to the non-LPCs **(Figure 24 A-B)**. However, the expression of other endogenous factors like Oct3/4 and Sox2 showed no significant difference in both LPCs and non-LPCs in both adult and foetal livers **(Figure 24 A-B)**.

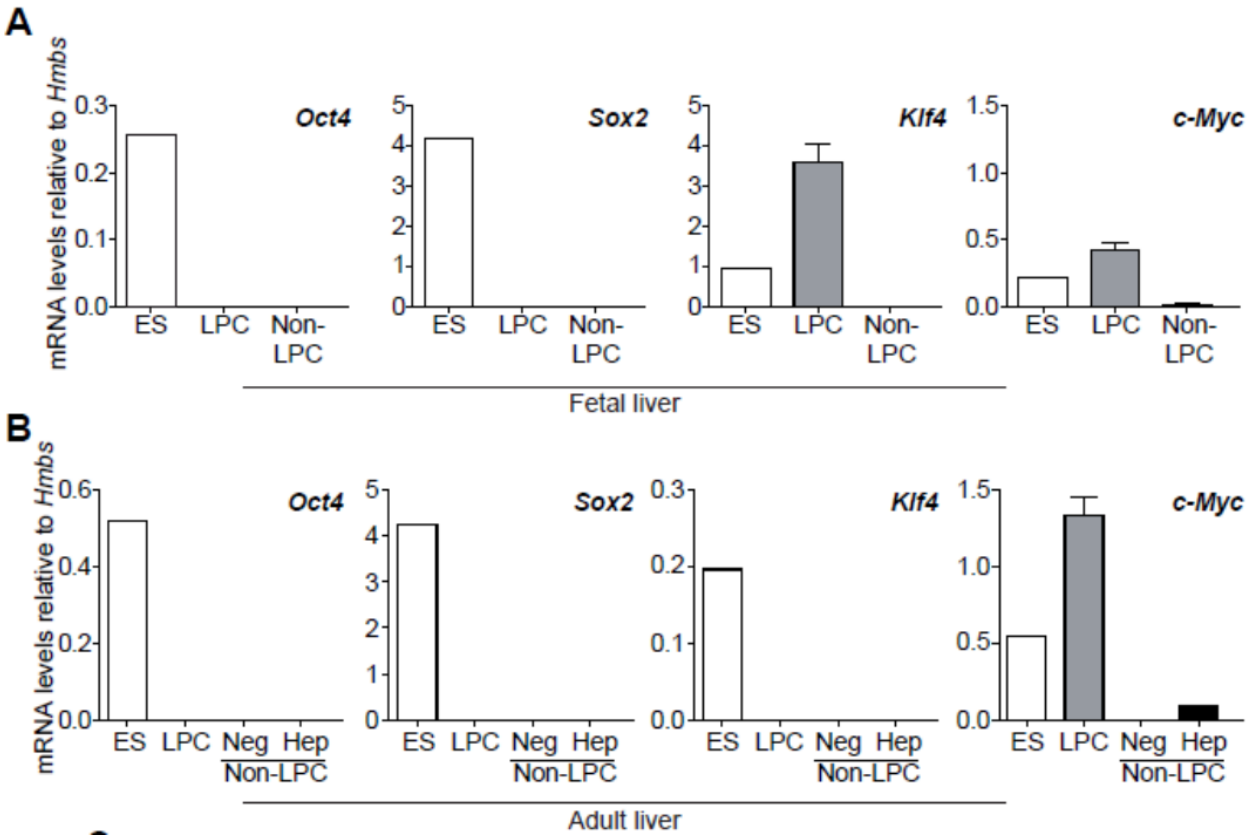


Figure 24 Expression of endogenous reprogramming factors and BAF complex factors in LPCs.(A) The bar graphs show the mRNA expression of the indicated reprogramming factors in embryonic stem cells (ES), foetal marker-positive LPCs and non-LPCs. (B) Bar graphs show the mRNA expression of the indicated reprogramming factors in ESCs, adult LPCs (marker positive), non-LPCs (marker negative cells) and hepatocytes.

8.9 Endogenous expression of BAF complex components determines increased reprogramming efficiency in LPCs

Having excluded that endogenous reprogramming factor expression could account for increased reprogramming efficiency, a reasonable explanation for the higher reprogramming efficiencies in LPCs compared to non-LPCs may be the progenitor cells share some genomic similarities with ESCs compared to the differentiated cells. To test this hypothesis, a whole transcriptome of the LPCs and non-LPCs was compared with the transcriptome of the ESCs. The results were analyzed using principle component analysis (PCA) and pairwise distance calculation. For the analysis all the expressed genes in the cell lines were investigated. In contrast with our hypothesis, both PCA and pairwise distribution calculation demonstrated that LPCs shared a greater transcriptional distance from the ESCs compared to the non-LPCs. This suggests that instead of common transcriptionally overlapping signature specific factors might account for **(Figure 25 A).**

An alternate explanation for the high reprogramming efficiencies of the LPCs can also be that other factors may contribute to enhance reprogramming such as chromatin remodelers like the BAF complex. Recently, it had been proved that the BAF complex can enhance the reprogramming process by transforming the heterochromatin of the differentiated cells to the euchromatin like state of the ESCs [176]. To gain insights into the molecular mechanisms lying behind the LPCs high reprogramming efficiencies, mRNA from freshly isolated LPCs and non-LPCs was eluted and quantitative polymerase reaction analysis was carried out to check the expression levels of BAF complex members Brg1 and Baf155. The expression levels revealed significantly higher levels of BAF complex members Brg1 and Baf155 in the LPCs compared to the non-LPCs in both adult as well as foetal liver cells **(Figure 25 B and C)**. Foetal LPCs showed nearly 10-15 fold higher levels of Baf155 and nearly 6-8 fold induction of Brg1 compared to the non-LPCs **(Figure 25 B)**. A similar induction of Brg1 and Baf155 was observed also in the LPCs derived from the adult mice livers compared to the non-

LPCs suggesting that conserved mechanism of the gene expression control may exist in foetal and adult liver stem/progenitor cells **(Figure 25 C).**

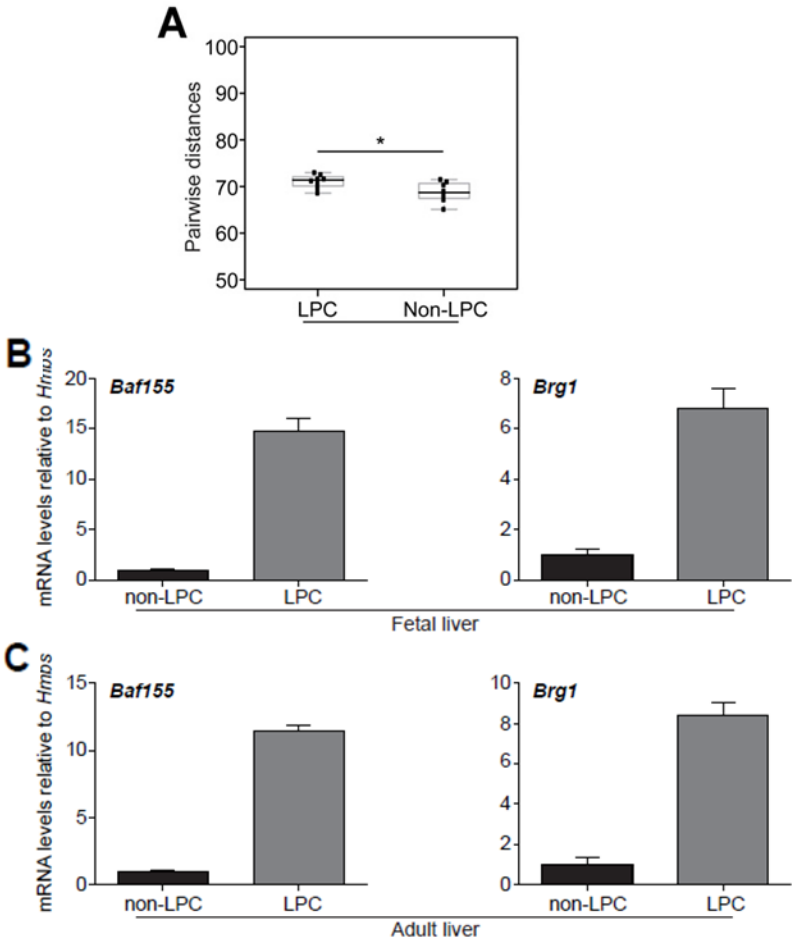


Figure 25 Expression of endogenous reprogramming factors and BAF complex factors in LPCs. (A) Pairwise distance calculation of LPCs and non-LPCs from established ES cell whole-transcriptome profiles. (B) The bar graphs show the mRNA expression of the indicated BAF factors (Baf155, Brg1) in foetal LPCs compared to non-LPCs (unsorted foetal liver). (C) The bar graphs show the mRNA expression of the indicated BAF factors (Baf155, Brg1) in adult LPCs compared to non-LPCs (percoll-purified hepatocytes). Expression of the indicated factors was set to 1 in non-LPCs.

8.9.1 Knock down of BAF complex components abolishes increased reprogramming in LPCs

Increased expression of the chromatin remodelling BAF complex in the respective target cells for reprogramming has been proven to increase the reprogramming efficiencies [176]. In LPCs, the two main components of the BAF complex namely BAF155 and Brg1 were found to be significantly elevated when compared to their differentiated counterparts. To assess the hypothesis that higher reprogramming efficiencies in LPCs are a consequence of Brg1 and Baf155 expression levels, a loss of function experiment was conducted using Lentiviral mediated shRNA transfer to knock down BAF155 and Brg1. The respective shRNAs were cloned into a SFFLV lentiviral vector and then transfected into Lenti-X cells for virus production. LPCs and non-LPCs were freshly isolated and the lentivirus was added to the cells. 12 hours post transduction, the OKSM reprogramming virus was added and the cells were reprogrammed with the same reprogramming protocol as mentioned earlier **(Figure 26 A)**. A scramble shRNA was used as a control for the experiment. 10 days post transduction with the reprogramming virus; the arising iPSCs colonies were stained for AP and documented. A strong reduction in the AP positive colonies in the LPCs was observed after the knockdown of the BAF complex members in both the adult and foetal derived LPCs compared to the scramble **(Figure 26 A, C)**. However, no significant difference was noted in the reprogramming of the non-LPCs compared to the scramble **(Figure 26 B, C)**. The knockdown experiment of the BAF complex members in the LPCs neutralised the higher reprogramming efficiencies observed in the LPCs compared to the non-LPCs suggesting that the higher reprogramming efficiencies of the LPCs were strongly associated with the presence of the BAF complex components in the LPCs.

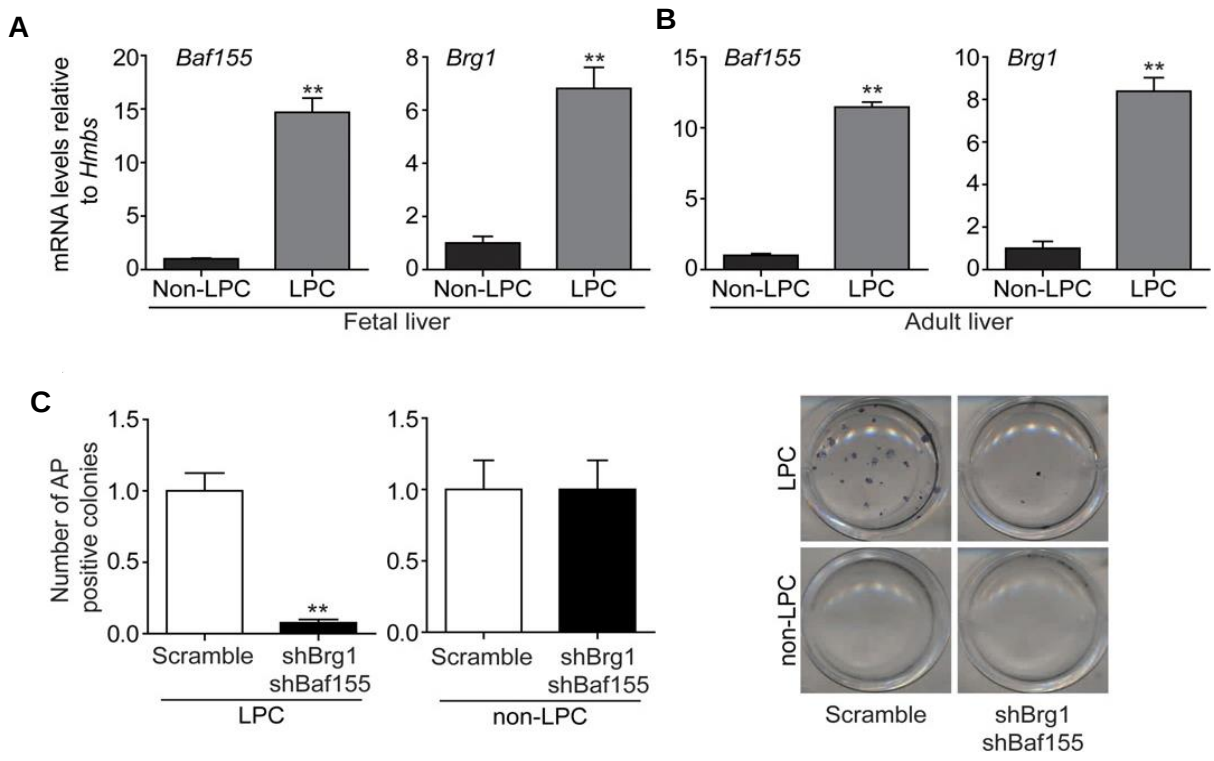


Figure 26 shRNA mediated knock down of Brg1 and Baf155. (A) The bar graphs show the mRNA expression of the indicated BAF factors (Baf155, Brg1) in foetal LPCs compared with non-LPCs (unsorted foetal liver). (B) The bar graphs show the mRNA expression of the indicated BAF factors (Baf155, Brg1) in adult LPCs compared with non-LPCs. (C) Colony numbers of scramble-infected cultures were set to 1 with respect to LPCs and non-LPCs. Representative AP-stained plates of shBaf155/shBrg1- and scramble-infected LPCs and non-LPCs after 10 days of reprogramming (right panel).

9 Discussion

9.1 Stem cells: An ultimate source for reprogramming

The current study is a step in the direction to identify the ultimate cell type for reprogramming that bypasses the current hurdles of low reprogramming efficiencies and long duration. Moreover, it helps to shed light on the mechanisms present in a particular cell type allowing their successful reprogramming with unusually high efficiency in a short time. As described in other organs such as blood and/or muscle, the differentiation stage of the mouse liver cells limits the reprogramming efficiency of these cells into iPSCs [1, 46, 140, 175]. A series of experiments allowed us to define via surface markers several cell populations within adult and foetal liver thereby setting the basis for an objective comparison of reprogramming efficiencies. To this we successfully showed that liver progenitor cells (LPCs) from both adult and foetal liver showed significantly higher reprogramming efficiencies when compared to the non-LPCs [1]. Intriguingly, increased reprogramming in LPCs compared to non-LPCs occurred independently of proliferation, transcriptional distance and “Yamanaka” factor expression but was dependent on the endogenous levels of certain chromatin remodelling factors. The study is first experimental evidence proving that freshly isolated progenitor cells from solid organs such as liver show significantly higher reprogramming efficiency compared to its differentiated counterparts independent of proliferation and the expression of endogenous pluripotency genes.

After the initial discovery of the iPSCs technique by Yamanaka and Takahashi, cell types of different origin and from different organs such as brain [177], hematopoietic system [4], muscle[178] and skin[179] were subjected to this process. Originally mouse embryonic fibroblasts were used in the first studies. In fact, MEFs have a primary mesenchyme origin and are quite heterogeneous as besides fibroblasts, endothelial cells, haematopoietic cells, muscle cells and also probably mesenchyme stem cells can be found within one preparation [137]. Apart from MEFs, cells of ectodermal origin such as neural stem cells were reprogrammed [135]. Finally also cells of endodermal origin were reprogrammed. Yamanaka and colleagues were the

first to report reprogramming of stomach and liver cells towards iPSCs [160]. In those studies it became more and more evident that the cell type of origin strongly influences the reprogramming efficiency. For e.g. B-cells require further factors expressed together with the Yamanaka factors [160]. Moreover, it has also been hypothesized that the cell type of origin can also have an impact on the reprogramming process with a bias in differentiation towards the respective lineage finally leading to epigenetic memory [136, 180]. Thus, the identification of less restricted cell subpopulations within a certain tissue which are more prone to reprogramming but also unbiased in terms of differentiation could prove to be a safer and better cell type for the generation of induced pluripotent stem cells.

Based on this hypothesis, several reports were published where progenitor or stem cell subsets of different organs were reprogrammed. The neural stem cells (NSCs) when subjected to reprogramming by different combination of reprogramming factors showed varying reprogramming efficiencies. However, the reprogramming efficiencies of the NSCs after the omission of c-Myc, Sox2 and Klf4 dropped to 0.011% compared to the 3.6% after 4 factor reprogramming [173, 174]. When compared to NSCs the reprogramming efficiencies of the fully differentiated post mitotic neurons was reported to be 0.8% with the 4 factor reprogramming system thereby strongly underpinning the phenomena that the differentiation stage might be the limiting stage for reprogramming in a solid organ like brain [175]. In the hematopoietic system, a more systematic approach was carried out by the use of secondary system to analyze the effect of differentiation stage on reprogramming [4]. Different subsets of hematopoietic cells were subjected to reprogramming from the mice generated from the iPSCs to make sure that every cell of the mice carries exogenous reprogramming vectors. It was observed that the reprogramming kinetics were much worse in the mature compartments with the overall reprogramming efficiencies of 0.02%-0.06% compared to the reprogramming kinetics and efficiencies in their undifferentiated counterparts (7%-28%) [4]. This hypothesis was also proven to be true in two other progenitor compartments namely the skin and the muscles [139, 178, 181]. Like NSCs, dermal papilla cells also express high endogenous levels of Sox2 and c-Myc which enabled their reprogramming using only Oct3/4 and Klf4 [139]. These cells were also

reprogrammed successfully only by the use of Oct3/4 [182]. However, the study lacked head to head comparison between the differentiated and the progenitor subset of cells. However, in the muscle system, the use of secondary system proved to be advantageous in demarcating the progenitor and more mature subset of the cells present in muscle tissue thereby providing a strong comparison between these two cell populations [178]. The study clearly described the superior reprogramming efficiency of the unipotent skeletal muscle precursors and bipotent non-myogenic mesenchyme progenitor (23%-29%) compared to differentiated myogenic compartment. However besides proliferatiive differences in the haematopoetic system, those studies did not provide mechanistcal insights into the differences in reprogramming between the progenitor and the more differentiated cell compartemnt.

In this study, the LPCs and hepatocytes were isolated based on the surface marker expression as described previously [1, 149, 183-186]. The cells were infected with equal amounts of polycistronic lentiviruses encoding 3 and 4 reprogramming factors. The reprogramming efficiencies of the LPCs were noted to be 22% and 30% compared to the 0.34% and 0.08% of the differentiated hepatocytes. A polycistronic lentivirus StemCCA reprogramming system was used in the study to generate iPSCs from murine LPCs and non-LPCs [119, 187]. Using this system, the reprogramming efficiencies of the mouse embryonic fibroblasts (MEFS) are reported to be around 0.5%-1% but the reprogramming efficiencies of liver derived progenitor cells were 10-100 folds higher than the previously reported efficiencies in any other progenitor cells of other tissues [188, 189]. The advantage of using this system over the retroviral system is the limited number of integration sites in the genome [119, 187].

The higher reprogramming efficiencies in the LPCs compared to the other stem cells/progenitor cells can prove to be a promising experimental system to implement fast and efficient reprogramming strategies in future, as the livers are routinely biopsied for the diagnosis of liver diseases. Future improvements in FACS purification and isolation of liver stem cells in mouse and men may help to increase the reprogramming efficiencies of the LPCs. A viral free generation of iPSCs from the LPCs in future might pave a path for the translation of the liver derived iPSCs to

human liver for curing liver diseases [84, 175]. LPCs can also exemplify a experimental setup to demarcate the novel mechanisms and molecular factors involved not only in reprogramming of stem/progenitor cells compared to their differentiated counterparts but also can also help in improving the reprogramming efficiencies in the future so that the iPSCs technology can be translated in future to humans. Given the intimate association between reprogramming, iPSCs and cancer formation such studies may also identify novel mechanisms of stem cell transformation related to the development of liver carcinoma.

9.2 Endogenous expression of reprogramming factors

Apart from the resemblance of the transcriptome to ESCs, another most promising attributes of adult stem cells or the progenitor cells is the expression of endogenous pluripotency factors. Along with their unique ability to maintain pluripotency, these factors also contribute to additional functions during cell differentiation and tissue development [190, 191]. In consideration with this, the endogenous expression of all the pluripotency factors was assessed. Klf-4 and c-Myc levels were noted to be significantly higher in LPCs compared to non-LPCs but this association was not explicit as Klf-4 levels were observed to be higher only in the foetal LPCs and not in the LPCs derived from adult murine liver but the reprogramming efficiencies were reported similar. The omission of the Klf-4 and c-Myc from the reprogramming cocktail failed to produce iPSCs thus proving that the endogenous levels of the Klf-4 and c-Myc were not attributing to the significantly higher levels of reprogramming in the LPCs. This result was in line with the previously published data on keratinocytes where the omission of Klf-4 from the reprogramming cocktail due to its endogenous expression failed to reprogram the cells successfully [179]. This presence of the pluripotency genes in progenitor compartments in some context can point to their non-pluripotency functions but in presence of exogenously expressed genes could serve as a help by changing their function to improve the process of reprogramming [1]. However, the impact of these endogenously expressed pluripotency genes on iPSCs

generation is poorly understood due to the lack of knowledge about the mechanism of the pluripotency itself [1, 46, 140, 173, 189, 192, 193].

9.3 Proliferation and reprogramming

Recently, it has been proved that increase in the reprogramming efficiency of a cell is a stochastic event and is directly proportional to its rate of cell proliferation because of the presence and expression of cell cycle associated genes like c-Myc present in the reprogramming cocktail [134]. Reprogramming was correlated with a change in cell morphology within one round of cell division after transduction with the reprogramming factors [194] . Thus it was proposed that every cell can give rise to an iPSCs and this stochastic event is thereby determined by latency and other mechanisms independent of proliferation. Thus, a cell type having similar cell cycle profile like the ESCs can reprogram faster than the other cell types thus shortening the latency [134]. The significantly higher reprogramming efficiencies in the LPCs also pointed to a pronounced acceleration in the overall reprogramming process giving rise to fully reprogrammed iPSCs colonies in the LPCs by day 10 after transduction. However, a systematic analysis comparing the reprogramming kinetics of the LPCs compared to the hepatocytes showed increased in the reprogramming efficiencies and kinetics occurred independently of the cell proliferation. To undermine the probability that the efficient reprogramming in the LPCs is proliferation dependent a mathematical curve fitting analysis was performed to calculate the latency for LPCs and their differentiated counterparts. A BrDU as well as PI based cell cycle assay was performed to check for the proliferation capacity of both the LPCs as well as the differentiated liver cells. More than 90 fold increase in reprogramming efficiency and 10 fold shortened latency in colony formation of LPCs was observed compared to the non-LPCs assuming that non-LPCs reach the plateau at the same time but the proliferation rates were only 15-20% increased in the LPCs compare to the non-LPCs. The doubling in the proliferation rates by a simulation to adjust the proliferation of non-LPCs still failed to make the curves collapse and match the latency of non-LPCs thus proving that

proliferation may not always be the factor increasing the reprogramming efficiencies of these cells. Similar result were obtained in the haematopoietic system [46].

c-Myc is a potent oncogene with dual role in reprogramming. Firstly, it suppresses differentiation associated genes and secondly, it activates genes which are present in highly proliferative cells like cancer cells, iPSCs and ESCs [195]. In liver progenitor cells, endogenous c-Myc levels exceeded the levels of ESCs and hence, c-Myc was omitted from the reprogramming cocktail. The reprogramming efficiencies dropped from 30% to 22% in LPCs after 3F reprogramming but virtually no difference was seen in the reprogramming kinetics. As c-Myc is known to be a potent driver of proliferation, omission of c-Myc from the reprogramming cocktail did not have any effect on the reprogramming kinetics pointed to a hypothesis that proliferation may not be the only factor attributing to increase reprogramming in some cells. The iPSCs colonies were assessed on the basis of the reactivation of the endogenous Oct-4 locus reporter allele [1].

9.4 Certain factors but not the transcriptome facilitate reprogramming

A possible theory explaining this trend could be the presence of adult stem cells or progenitor cells which reside in the adult tissues and help in the repair and maintenance of these organs and tissues share some characteristics of the ESCs like self renewal and differentiation [190]. Thus, it has been hypothesized that the closer the relation of the target cells to the stem cell or embryonic stage is the more effective is the reprogramming process. This can be attributed to the point that the epigenome of the stem cells may closely resemble ESCs when compared with somatic organ cells [191-194]. However, stem cells have different microenvironments or stem cell niches and these niches can exhibit large variation in their differential potential and status. These variations can influence the proximity of the stem cell to ESCs thereby concerning on the basis of their naivety, proliferation capacity and epigenetic memory [190, 195]. Following in line with the above mentioned hypothesis, LPCs isolated from both adult and foetal murine liver contained a subpopulation of bipotent progenitor

cells that self-renewed under continuous passages and also were able to differentiate into hepatocytes as well as cholangiocytes [149, 183]. This subpopulation also enclosed a small unipotent cell population which showed restricted differentiation potential and expansion capacity [157]. The LPC population thus is not a defined stem cell population but is a progenitor enriched population within the liver compartment. However, the current protocols are not sufficient to isolate the unipotent population within the liver progenitor state and hence the reprogramming hierarchy in this population still remains a question.

The significantly higher reprogramming efficiencies of LPCs compared to the non-LPCs intrigued in reasoning of the two main hypotheses: (i) a globally shorter distance between the target cell and the ESCs help in the enhancement on reprogramming efficiency and (ii) whether cell intrinsic mechanisms apart from cell proliferation take part in the process of reprogramming. The above study tried to address these questions in a systematic approach. In the first case, to test the possibility of similarity in the transcriptome of the liver progenitor cells with the pluripotent cells, a principle component analysis (PCA) and pairwise distance calculation was on all expressed genes was investigated in all the cell lines. Surprisingly, the transcriptome of the LPCs showed a greater distance from the ESCs than the non-LPCs [1]. The lower proximity between LPCs from the ESCs transcriptome compared to the non-LPCs reflect on the fact that even though the LPCs may not share the signatures of the ESCs, there can be different molecular regulatory pathways which can mediate the reprogramming process in these cells. There is also a possibility that the differentiation stage of the target cell and the endogenous cell factors act in concert to revert the epigenome of the reprogramming cell back to the pluripotent cell stage. However, a more in depth study is required to prove the above mentioned hypothesis and as of now the matter remains as a speculation due to the lack of knowledge.

9.5 The BAF complex

Adult stem cells are generally more euchromatin rich while differentiated cells are more rich of heterochromatin [196-199]. The BAF complex has been hypothesized to mediate reprogramming by regulating DNA methylation either by directly recruiting DNA methylases or by indirectly altering the expression of protein that can affect DNA methylation [200-202]. It can also play an important role by regulating Stat3 signalling thereby controlling LIF and Bmp4 pathways which control self renewal and maintain pluripotency [202]. Previously reported data suggest the role of Brg1, a BAF complex member in nuclear reprogramming and the knockdown of the Brg1 lead to the decrease in the levels of Oct3/4 [200, 201, 203]. The other component of BAF complex, namely Ini1 interacts with the reprogramming factor c-Myc thereby mediating reprogramming [201, 202, 204, 205]. The Baf155 component of the BAF complex, observed significantly higher in the liver progenitor cells compared to their differentiated counterparts is known to replace the endogenous Baf170 present already in the cells thereby converting the endogenous BAF complex to the esBAF complex which can also enhance and accelerate the reprogramming [176]. This underlines the role of BAF complexes in pluripotency and also in reprogramming by interacting with the binding sites of different transcription factors provided during reprogramming and also by converting the heterochromatin to the euchromatin state.

Previously, the esBAF complex components have ascribed the role of achieving a euchromatin rich state thus enhancing the binding of specific transcription factors to their respective DNA loci. These transcription factors could include the different reprogramming factors which can have easy access to their pluripotency gene promoters due to the presence of esBAF complex [176, 206-208].

Thus, the BAF complex members may define as the novel cornerstones of somatic cells which can contribute to the increased reprogramming capacity of the adult stem cells. In context of the above mentioned study, the BAF complex members are known to be expressed in various self-renewing tissue cells like the liver and the expression of Brg1, a BAF complex member is known to decrease as the cells differentiate giving rise to the adult liver [206, 209]. In line with these data, the LPCs showed significantly

higher levels of both the BAF complex members Brg1 and Baf155 compared to the differentiated cells of the liver. To verify whether the BAF complex is promoting these higher reprogramming efficiencies in the LPCs, both the BAF complex members were knockdown from the LPCs using a shRNA based lentiviral approach. The knockdown of the BAF complex members led to significant decrease in the iPSCs colony formation in the LPCs and the reprogramming efficiencies were similar to those observed in the differentiated liver cells [1]. The above mentioned result pointed towards the possible mechanism of the BAF complex in promoting the stronger reprogramming in the LPCs. The reanalysis of recently published transcriptome data on liver progenitor cells isolated from adult mice confirmed the hypothesis laid down in the thesis that increased levels of BAF complex observed in the liver progenitor cells compared to the differentiated cells may help in enhance reprogramming by changing the chromatin in these cells.

In summary, the above results point out towards a model where the BAF complex members may govern the transcription of the liver specific genes such as albumin thereby helping in the differentiation or regeneration of the liver. However, in the presence of reprogramming factors, the various components of the BAF complex act in concert with these transcription factors, thus converting the endogenous BAF complex to the esBAF complex state. This conversion of BAF complex to esBAF complex mediates the change of heterochromatin to euchromatin stage thereby promoting the binding of the ES cell specific transcription factors to their respective loci thus enhancing the reprogramming in the process.

To conclude, the above thesis clearly sums up a conclusion that the epigenetic status of a particular cell type facilitates the reprogramming efficiencies by creating favourable epigenome for the binding of stem cell genes. It also points towards a fact that as adult stem cells generally have a euchromatin rich state [196-199, 210] they may express higher levels of BAF complexes and can pose to be an ultimate cell type for fast and efficient reprogramming in the future.

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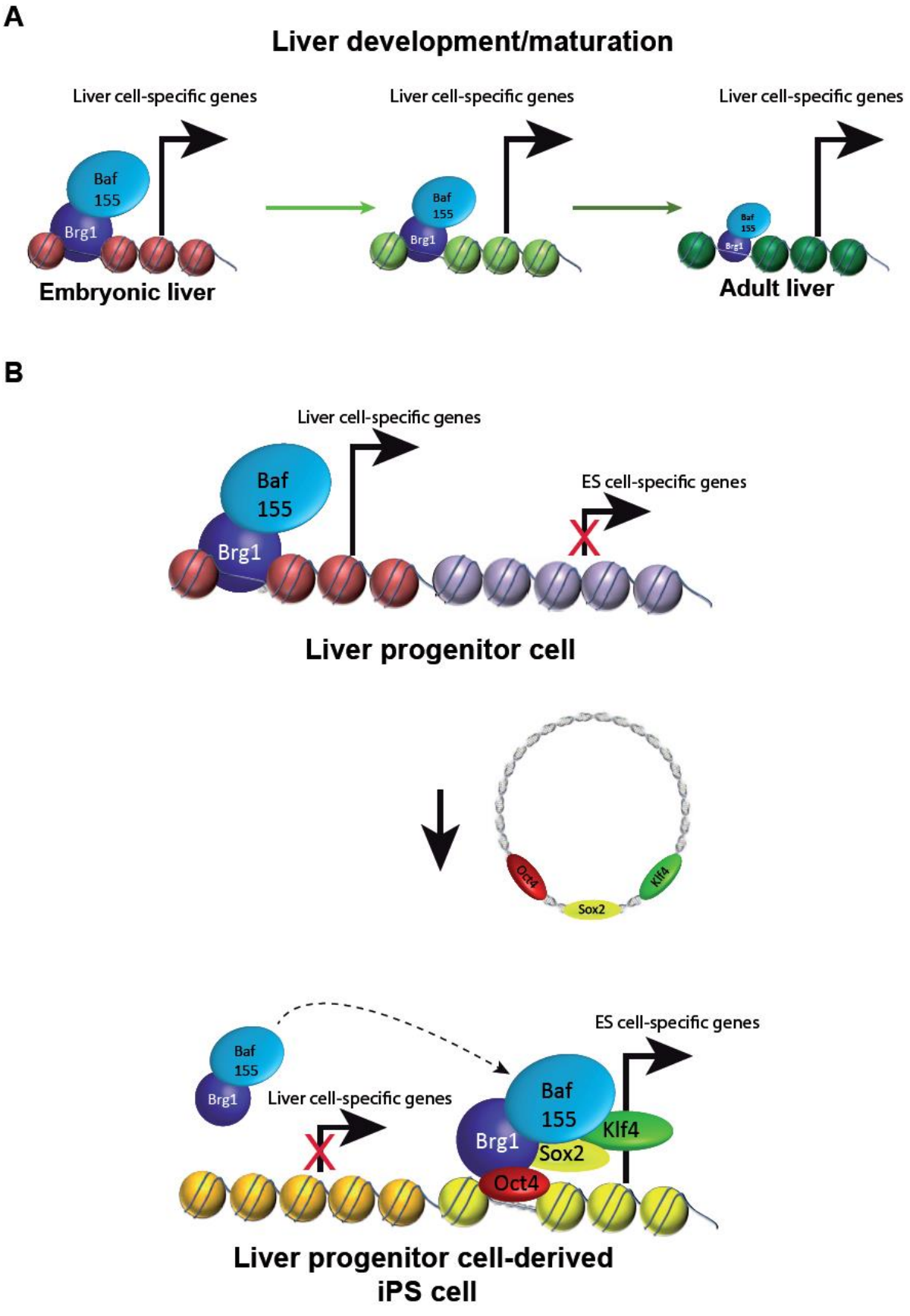
A
Liver development/maturation
Liver cell-specific genes
Liver cell-specific genes
Liver cell-specific genes
Baf 155
Brg1
Embryonic liver
Adult liver
B
Liver cell-specific genes
ES cell-specific genes
Liver progenitor cell
Oct4
Klf4
Sox2
ES cell-specific genes
Liver cell-specific genes
Liver progenitor cell-derived iPS cell

Figure 24. **A proposed BAF complex expression during liver development and liver progenitor cell reprogramming.**

1 A proposed BAF complex expression during liver development and liver progenitor cell reprogramming.

(A) Assumed model of BAF complex function (as reported for Brg1) during liver development. Particularly, the levels of Brg1 are decreased during liver development showing a decline with the differentiation stage from foetal to adult liver (illustrated by the smaller becoming BAF complex symbols) thus attributing its function to maintain liver differentiation/maturation/regeneration. The euchromatin is showed in red colour (for foetal liver), bright-green colour (for maturating foetal liver), or dark-green colour euchromatin (for adult liver) at liver-specific gene loci is shown. (B) A proposed model of endogenous BAF complex function during reprogramming of liver progenitor cells. BAF complex members govern the transcription of liver/LPC-specific genes which keeps the specific chromatin regions in an open state. Red euchromatin allows transcription of liver specific genes while violet heterochromatin prevents the transcription of ES-cell specific genes such as Oct3/4. In presence of the reprogramming factors (mirrored by the expression plasmid encoding Oct3/4, Sox2 and Klf4), BAF complex members switch their function (dotted arrow) to promote an ES-cell specific transcription by enhancing binding of the reprogramming factors to key reprogramming gene promoters. In reprogrammed LPC-iPSCs dark yellow represents heterochromatin at liver specific loci while bright yellow represents euchromatin at ES-cell specific loci (modified according to [175, 176].

10 Outlook

Since 2006, direct reprogramming of the somatic cells into induced pluripotent stem cells has been accomplished to circumvent the ethical issues revolving the use of the ESCs therapeutically as well as in basic research. The over-expression of four Yamanaka factors namely Oct-4, Sox2, c-Myc and Klf4 reprograms the fully differentiated somatic cells into iPSCs which are molecularly and epigenetically similar to the ESCs and hence can differentiate into any cell type. iPSCs thus holds a promising future in clinics and also in basic research. However, the use of iPSCs still encompasses major safety concerns for clinical applications due to the integration of viral vectors in the genome as well as lower reprogramming efficiencies. It remains under debate whether iPSCs could ever represent a valuable therapeutic tool directly used for the treatment of diseases such as Parkinson's and diabetes. The true value of iPSCs may instead lay instead in the modelling of diseases or in the delineation of basic molecular programs controlling cell fate decisions. A deeper understanding of the latter programs could ultimately be used for the in vivo programming of cells to trans-differentiate cells within an organ for therapeutic processes, eg the trans-differentiation of exocrine pancreas into endocrine pancreas for the treatment of diabetes mellitus.

Generation of induced pluripotent stem cells from tissue stem/progenitor cells such as blood, nervous system, muscle or liver can pose to be remarkable cell source as they possess various advantages over their differentiated counterparts. It was well observed that the tissue stem/progenitor cells show a higher reprogramming efficiency compared to the differentiated cells including MEFs. This makes the tissue cells a faster and efficient source of reprogramming. Additionally, blood cells and liver cells are easily accessible due to regular blood donations and liver biopsies in the clinics.

The origin of the donor cell population determines the reprogramming efficiency and its differentiation potential and thus the cells derived from different tissue types show different tendencies in terms of the reprogramming efficiencies and also show an inclination in differentiation towards the cells of the same origin. This fact can be attributed to the presence of the epigenetic memory in these cells. The cells derived

from the same niche also show a typical epigenetic status, specific gene expression profile and presence of certain levels of endogenous reprogramming factors.

Despite the rapid progress in the field of reprogramming over the last half decade, the molecular mechanisms underlying the process of fast and efficient reprogramming are still not fully understood. It is of utmost importance to substantiate the various mechanisms underlying the process of reprogramming to generate iPSCs that are indistinguishable from the ESCs to be used clinically. Until these questions have been resolved, ESCs will remain an important reference and gold standard for pluripotent cells.

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