Induced pluripotent stem (iPS) cells from human fetal stem cells

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ABSTRACT

Pluripotency defines the ability of stem cells to differentiate into all the lineages of the three germ layers and self-renew indefinitely. Somatic cells can regain the developmental potential of embryonic stem cells following ectopic expression of a set of transcription factors or, in certain circumstances, via modulation of culture conditions and supplementation with small molecule, i.e. induced pluripotent stem (iPS) cells. Here, we discuss the use of fetal tissues for reprogramming, focusing in particular on stem cells derived from human amniotic fluid, and the development of chemical reprogramming. We next address the advantages and disadvantages of deriving pluripotent cells from fetal tissues and the potential clinical applications.

KEYWORDS

Reprogramming; small molecules; fetal stem cells; induced pluripotency; transcription factors; translational medicine; cellular therapy.

A. Rejuvenating somatic cells to pluripotency.

Fetal cell fate is reversible chemically. We previously reprogrammed human fetal stem cells to functional pluripotency using valproic acid (VPA) and without genetic manipulation or ectopic expression of pluripotency factors (1-3). Somatic cells can be reprogrammed into pluripotent stem cells, i.e. induced pluripotent stem (iPS) cells, to regain the developmental potential of embryonic stem (ES) cells, including the capacity to self-renew indefinitely and to differentiate into all the lineages of the three germ layers. Rejuvenated cells are attractive tools in biomedical research to study development and disease, and in regenerative medicine to produce patient-specific cells that can be injected without immunosuppressant. Pioneered by Yamanaka's group in 2006-7, iPS cells were first generated by retroviral transduction of a combination of four transcription factors, i.e.OCT4, KLF4, SOX2 with or without c-MYC (4-6) or OCT4, SOX2, NANOG with or without LIN28 (5, 6). Various reprogramming strategies have since been developed to increase reprogramming efficiency and create iPS cells free of exogenous DNA (footprint-free) with the purpose of avoiding tumorigenic insertional mutations and reactivation of transgene expression during differentiation, which represent a major hurdle for iPS cells applications. Integration-free techniques use for example minicircle DNA, transposons, RNA viral vectors, acid-free methods, and episomal vectors, with episomal reprogramming being at present the most efficient method and the most likely translatable technology for producing clinical-grade human iPS cells via ectopic expression of transcription factors (7). Manipulation of the environment, such

as biophysical cues, can also modulate cell fate (8). Similarly, chemicals, in particular small molecules, have been used to modulate reprogramming, either by increasing reprogramming efficiency, and/or to replace one or more transcription factors (9). Several small molecule compounds involved in epigenetic regulation have been identified for their role in the reprogramming process either via somatic cell nuclear transfer (SCNT), or ectopic expression of a define set of transcription factors to replace one, or more, of the reprogramming factors. For example, it was shown that hyperacetylation improves reprogramming of somatic nuclei following nuclear transfer (10) and that the histone deacetylase inhibitor trichostatin A (TSA) reduces abnormal DNA hypermethylation following cloning by SCNT, thereby increasing success rate of mouse cloning via SCNT (11). Similarly, the histone deacetylase inhibitor valproic acid improves reprogramming efficiency of mouse embryonic fibroblasts (MEF) without the introduction of c-Myc (12) and enables the reprogramming of primary human fibroblasts with only two factors, Oct4 and Sox2, without the need for the oncogenes c-Myc or Klf4 (13, 14). Generally used for the treatment of epilepsy and bipolar disorder, VPA (2-propyl pentanoic acid) enhances OCT4 promoter activity via activation of the PI3K/Akt/mTOR pathway in mouse cells (15, 16). VPA is involved in several regulatory mechanisms including GSK3b, Akt, ERK, phosphoinositol, tricarboxylic acidcycle, GABA, and OXPHOS pathways (17).

Induced pluripotent stem cells, which can be generated from different tissues, have properties that differ according to their tissue of origin, because they maintain a unique residual DNA methylation that influences their

differentiation potential, i.e. “epigenetic memory of the donor tissue” (18). In addition, the tissue of origin can also influence the efficiency and yield of reprogramming. For example, keratinocytes being reprogrammed more readily than fibroblasts (19, 20), and fibroblasts isolated from different tissues vary in their reprogramming efficiency. In that context, stem cells, which are less lineage committed than terminally differentiated somatic cells, are easier to reprogram, and can be reprogrammed with a reduced number of factors, and adult neural stem cells can revert to functional pluripotency using OCT4 only (21). In addition to the differentiation status of the tissue of origin, the development age of the parental population also influences the capacity of the cells to revert to an earlier state of pluripotency. For example, embryonic and fetal tissues can be reprogrammed with a higher efficiency than adult tissues, yielding iPS cells that are functionally most comparable to embryonic stem cells (18, 22, 23). Of importance, the *Ink4/Arf* locus, which encodes p15(INK4b) , ARF, and p16(INK4a) genes in human chromosome 9p21, has been shown to be a barrier for iPS cell derivation, with the locus being silenced in both murine and human iPS and ES cells (22). The expression level of the Ink4/Arf locus, which is progressively upregulated in older cells and in cells from late developmental stages, inversely parallels reprogramming efficiency, explaining why older cells are more difficult to reprogram. Indeed, embryonic and fetal tissues can be rejuvenated using a reduced number of transcription factors. For example, human neural stem cells (NSCs), $CD34^+$ amniotic fluid cells, and keratinocytes, as well as mouse fibroblasts have been reprogrammed with only OCT4, either alone (24, 25), or in conjunction with a small molecule cocktail (26, 27). Small molecules are

advantageous because they are not expensive, non-immunogenic, cell-permeable, easily synthesized, and target specific pathways. Complete chemical reprogramming in the absence of genetic manipulation has been overcome in mouse epiblast stem cells, which already express pluripotency markers oct4, sox2 and nanog but are incapable to contribute to chimerism, using a combination of small molecules to simultaneously inhibit LSD1, ALK5, MEK, FGFR and GSK3 (28). More recently, mouse embryonic fibroblasts have been fully reprogrammed to pluripotency using only small molecule compounds (29).

B. Fetal tissues as a source for reprogramming

Mesenchymal stem cells (MSCs), originally isolated from the bone marrow but found in various prenatal and postnatal tissues at different stages of development, are plastic-adherent cells with clonogenic capacity characterized by the co-expression of CD73, CD105 and CD90, absence of expression of hematopoietic markers as well as pluripotency markers, their ability to differentiate *in vitro* to adipogenic, chondrogenic, and osteogenic cells. MSCs do not form teratomas following subcutaneous injection in immune-compromised mice. This definition complies with the minimal criteria for defining multipotent MSCs according to the International Society for Cellular Therapy position statement (30). MSCs have generated substantial interest for use in cell therapy and tissue engineering due to their ability to migrate to sites of injury and regenerate and repair damaged tissues (31, 32).

Functionally, MSCs are metabolically active, producing cytokines and growth factors. They have immunosuppressive properties, suppressing T cell proliferation and dendritic cell maturation, reducing B cell activation, and polarizing macrophages from a M1 pro-inflammatory to a M2 anti-inflammatory type in response to acute injury and/or inflammation (33). Fetal MSCs can be isolated from fetal tissues, such as liver, bone marrow, and 1st trimester blood (34, 35), as well as amniotic fluid (36, 37) and placenta (38). We, and others, have shown that MSCs isolated from fetal tissues show advantageous characteristics compared to their adult counterparts, including smaller size, active telomerase with longer telomeres, faster kinetics and greater expansion capacity, but do not self-renew indefinitely. They also show a unique adhesion molecule profile and broader differentiation potential, although they are not pluripotent, i.e. have the capacity to differentiate into all the lineages of the three germ layers. For example, human fetal blood MSCs and first trimester chorionic stem cells (CSC) transplanted perinatally in a mouse model of osteogenesis imperfecta reduced considerably long bone fracture rates and bone brittleness, and increased bone volume, with donor cells engrafting at sites of bone formation and differentiating into functional osteoblasts, producing the Collagen Type 1 alpha 2 chain which is absent in non-transplanted mice (39-41). Similarly to other fetal MSCs, human c-Kit+ amniotic fluid stem cells (AFS cells) show broader differentiation capacity, co-expressing CD73, CD90 and CD105 but not hemoatopoietic markers or pluripotency markers (37). We demonstrated there that despite sharing 91.6% RNA transcriptome identity with human embryonic stem cells, human first and second trimester AFS cells do not express most markers of

pluripotency and are not pluripotent, failing to form teratomas *in vivo,* although they show evidence of lineage priming. However, along with placental chorionic MSCs (CSCs), AFS cells are abundant, readily available and easily accessible, being collected at mid-gestation using minimally invasive technique during prenatal diagnosis or fetal treatments. Similar to other fetal stem cells, AFS cells have a small size, high growth kinetics, possess an active telomerase, and a more primitive phenotype. They also provides the opportunity to generate “younger” pluripotent cells, i.e. with reduced number of acquired mutations and longer telomeres, providing unprecedented opportunity to derive patient-specific iPS cells for prenatal and perinatal use, whilst avoiding the risk of immunorejection and the need of immunosuppressant. Because they are isolated from fetal tissues, fetal MSCs may have accumulated less genetic damage or somatic mutations than older cell types.

Deriving iPS cells from peripheral blood (PB) is convenient and less invasive than from dermal fibroblasts, where several weeks are required to establish a primary cell culture from skin biopsy. Fetal HSCs can also be derived at birth from the umbilical cord blood (UCB). Around 1% of the cells isolated from UCB expresses the CD34 surface marker, and are negative for CD38. The frequency of CD34+ cells in cord blood is higher than that of adult BM or peripheral blood following cytokine mobilization (42), and compared with BM cells, CD34+ UCB cells proliferate more rapidly and generate larger numbers of progeny cells (43). Besides, it was demonstrated that cord blood HSCs express neuronal proteins and can differentiate into neuronal-like cells or glial

cells (44). Induced pluripotent stem cells derived from human cord blood have recently been shown to generate a large number of functional neurons including dopaminergic cells, which may serve for the development of novel regenerative treatment strategies (45).

C. Chemical reprogramming of human fetal stem cells isolated from first and second trimester amniotic fluid

We previously used microarray-based transcriptome analysis to investigate whether first and second trimester AFSC show similar gene expression profiles and reported that both populations are related but differ with regards to cell size and molecular signature, with a cell-specific gene expression signature comprising 366 genes for first trimester and 340 genes for second trimester AFSC (37). Residual levels of OCT4 can be deteced in both first and second trimester AFS cells, whilst SOX2 is only expressed in first trimester AFS cells, when the cells are expanded on plastic at 37 1C with 5% CO2 atmosphere, either in alpha minimum essential medium (a-MEM) containing 15% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin supplemented with 18% Chang B and 2% Chang C (36); in Dulbecco's Modified Eagle's Medium high-glucose (DMEM) containing 10% FBS supplemented with 2 mmol/l L-glutamine, 50 IU/ml penicillin, 50 mg/ml streptomycin (1, 3). However, expression levels are relatively low compared to those found in ES cells, i.e. <1% for OCT4 and <10% for SOX2, albeit expression is homogeneous, as shown by confocal microscopy and flow

cytometry. We hypothesized that low expression levels of OCT4 and SOX2 might reflect the susceptibility of the cells to revert to an earlier state of pluripotency under appropriate culture conditions and without the need of exogenous expression of transcription factors. We found that culturing the cells on conditions designed to sustain the pluripotent phenotype of hES and iPS cells, i.e. culture on Matrigel-coated plastic plates in low growth factor hES cells feeder-free culture medium, increased levels of expression of OCT4 and SOX2, and that supplementation of the culture with 1 mM VPA for 5 days, followed by culture on Matrigel in iPS cell medium fully reverted the cells to functional pluripotency at a clonal level, as evidenced by their capacity to form embryoid bodies *in vitro* and teratomas *in vivo*, both containing cells expressing markers of the three germ layers, such as neural tube and squamous epithelium (ectoderm), collagenous tissue and blood vessels of human origin (mesoderm), and alveolar and gut epithelium (endoderm). Reprogrammed cells homogeneously co-express Tra-1-60, DNMT3b and REX1, which satisfies the stringent criteria for human fully-pluripotent cells (46). Human iPS cells derived from both first and second trimester AFS cells expanded over 8 weeks following reprogramming maintain genetic stability, protein-level expression of key pluripotency factors, high cell-division kinetics (21 ± 3.4 h population doubling), active telomerase activity, and capacity to differentiate into lineages of the three germ layers, such as definitive endoderm, hepatocytes, bone, fat, cartilage, neurons and oligodendrocytes. Some cells organized in embryoid body structures were observed contracting rhythmically in synchrony, mimicking a primitive cardio sphere appearance. VPA-induced iPS cells showed repression of X-inactivation (as seen by

absence of expression of the X-linked Xist gene), with expression of a subset of X-linked genes in the gene arrays showing that CXORF15, PLS3, RBBP7, and UTX being expressed, confirming repression of X-chromosome inactivation in AFS-CiPS cells. Of interest, although the transcriptome profile of chemically reprogrammed AFS cells clearly separate from those of their parental lines, with an overlap between AFS-CiPS cells and hES cells of 7572 genes, among which 273 genes associated with WNT and NOTCH signaling pathways were only expressed in iPS and hES cells but not in the parental population, AFS-CiPS cells are not identical to hES cells, with 15% of hES cells genes not being expressed in CiPS cells (1). However, similarly to hES cells but contrary to the parental line, AFS-iPS cells express tripartite motif protein 28 (TRIM28), also known as Transcription Intermediary Factor–1 beta(TIF1b) or KRAB-Associated Protein 1 (KAP1), which is a specific and indispensable factor for the maintenance of pluripotency in hES cells (47). Interestingly, we showed that second trimester AFS cells, which do not express SOX2, can be efficiently reprogrammed using a similar protocol (3), albeit abolishing OCT4 expression in both first and second trimester AFS cells prevented VPA-mediated reprogramming, as seen by the absence of Tra-1-60+ cells (personal communication, unpublished data). These results indicate that OCT4, but not SOX2, is indispensable for the chemical reprogramming of human somatic cells using VPA. The residual expression of OCT4 in amniotic fluid stem cells raised the possibility that AFS cells might originate from a founder population of stem cells of epiblast origin such as primordial germ cells, which may have been retained in the amniotic fluid early during their migration to the genital ridges. The epiblast origin of AFS cells was further

documented during whole genome transcriptome analysis by high expression of *FGF5*, a marker of pluripotent epiblast cells which is not expressed in the inner cell mass (ICM). The transcriptome of AFS-iPS cells showed absence of expression of markers which constitute the NCSC molecular signature, such as *FOXD3* and *RET*, indicating that AFSC might be of primordial germ cells/progenitors origin (1). Interestingly, we showed that AFS cells express the PGC markers *c-KIT*, *T*, *FGF8*, *SOX17*, *STELLA*, *DAZL*, *NANOS2*, *NANOS3*, *VASA*, *SSEA1*, *FRAGILIS* and *PUM2* but not *ERAS*, which is repressed in PGC (48). At the protein level, the AFS cell population contains cells positive for FRAGILIS, SSEA1, TNAP, NANOS, BLIMP1, PUM2, STELLA, DAZL and VASA. Interestingly, SSEA1, which is expressed in human PGC but not in hES cells, is expressed in AFS cells. Tissue Non-Specific alkaline Phosphatase (TNAP) is strongly expressed in the cytoplasm; as well as BLIMP1, a transcriptional repressor expressed in the nucleus of PGC which progressively relocates to the cytoplasm during migration of PGC to the genital ridge but is absent in EG cells (49); with FRAGILIS and STELLA being expressed in both nucleus and cytoplasm of AFS cells, as is the case for PGCs (50). NANOS and DAZL, known to relocate from the nucleus to the cytoplasm in human fetal germ cells and PUM2 (51), are expressed in the cytoplasm of AFS cells. In migratory PGC, this relocation with parallel down-regulation of OCT4 and the onset of expression of VASA, which in AFSC is expressed in the cytoplasm, indicating that AFSC might be related to PGC, which have been retained in the amniotic fluid early at the start of their migration to the genital ridge.

D. Advantages and disadvantages of deriving iPS cells from fetal tissues

Cells isolated from fetal tissues can be used for both prenatal and perinatal autologous and allogeneic therapy. However, the isolation of fetal cells from fetal somatic tissues in the first trimester requires pregnancy termination, an obstacle to autologous applications. The harvest of fetal blood in continuing pregnancies is feasible, but technically challenging. One group has developed ultrasound-guided fetal blood sampling in ongoing pregnancies at risk of haemoglobinopathies with a loss rate of only 5% at gestations as early as 12 weeks (52). Thin-gauge embryo-fetoscopes also allow early fetal blood sampling from the umbilical cord under direct visualization (53). Alternatively, amniotic fluid stem cells can be isolated during ongoing pregnancy from the surplus of amniocentesis, but this is not a prenatal diagnostic systematically performed. Most suitable fetal tissues include term placenta, which is discarded at birth and can be stored to isolate stem cells. We, and others, have shown that term placenta contains chorionic stem cells (CSC) with a similar phenotype to AFS cells (38).

Relevant to cell therapy, MSCs isolated from fetal tissues are more advantageous than their adult counterparts, including their more primitive phenotype, unique adhesion molecule profile, active telomerase, and smaller size. The multi-organs engraftment and therapeutic potential of human first trimester MSCs following prenatal and neonatal cell therapy has been documented in a number of animal models of human pathology, as well as in

humans (54). Of concern, there are several key hurdles that limit their translational potential: (a) the rarity of the cells and heterogeneity of the population, which necessitates *in vitro* cell expansion, (b) their isolation requires invasive procedures (c) they undergo replicative senescence, limiting the yield of primary cells that can be expanded *in vitro*, and (d) the rapid decrease in differentiation potential during passaging of primary cells. These changes in phenotype following *in vitro* culture hinder the development of effective therapies for long-term treatment, and highlight the need for new cell sources of MSCs for clinical applications. It is now possible to produce high yields of MSCs from pluripotent stem cells, replacing the need to use primary MSCs isolated from fetal tissues. The rationale behind replacing primary MSCs by MSCs derived from iPS cells is that *in vitro* cell expansion is not performed on primary MSCs but on self-renewing pluripotent stem cells, to bypass replicative senescence (55). Deriving MSCs from fetal iPS cells produced from amniotic fluid or placenta during ongoing pregnancy offers the possibility to generate cells with a fetal, as opposed to adult, MSC phenotype from non-aborted tissues.

Recent studies indicate that MSCs produced from iPS cells have some advantages over primary MSCs. For example, Lian et al (56) induced human iPS cells to MSC differentiation with a clinically compliant protocol and showed the resulting MSCs attenuated severe hind-limb ischemia and promoted vascular and muscle regeneration following transplantation into mice, with the benefits of iPS-MSCs on limb ischemia being superior to those of primary adult bone marrow MSCs. The authors postulate that the greater

potential of iPS-MSCs may be attributable to their superior survival and engraftment after transplantations. Importantly, MSCs derived from pluripotent cells (ES) also display immunomodulatory properties, displaying a unique immunophenotypic properties, with a smaller size and >30,000 fold proliferative capacity than bone marrow-derived MSCs (57).

E. iPS cells derived from fetal tissues for clinical applications

Generating iPS cells for clinical application aims at avoiding the potential risk of insertional mutagenesis associated with the random integration of the transgenes within the genome. Attempts to overcome this led to the development of excisable vectors, using Cre-recombinase to remove the integrated construct after reprogramming. However, each excision event leaves a small long terminal repeat fragment behind, which can still cause genotoxicity (58-60). An alternative is the *piggyBac* transposon system, where the transposase can be used after reprogramming to completely remove the transposon (61, 62). Nevertheless, the efficiency of this system is low, and DNA sequencing is required to screen for any genetic modifications at the insertion sites, making this technology too laborious to be used in a clinical setting. Non-integrating systems were developed to overcome the pitfall of insertional mutagenesis and produce safer, clinically relevant iPS cells. These include both virus-based and virus-free approaches. Non-integrating viral methods utilize adenovirus- or Sendai virus-based systems to transfect plasmids carrying the transgenes of interest (63). However, these

methods are hindered by even lower efficiency levels of around 0.001%, due to transient expression of the transgenes. Subsequently, another method used to achieve non-integrative transgene expression used episomal constructs (64) or mini-circle vectors (65, 66). Both are replicated once at every cell cycle under drug selection conditions, but once reprogramming is completed and drug selection is withdrawn the episomes are gradually lost, leading to transgene-free iPS cells. Despite this clear advantage, this method requires the use of the oncoprotein SV40LT which, combined with low efficiency, makes the protocol unsuitable to clinical use, particularly due to the laborious and time-consuming analysis required ensuring that the generated iPS cells are free of vector-derived sequences. A safer approach is the use of RNA-based technology (28), albeit efficiency remains low, and the technique is difficult to reproduce (for a complete review refer to (67). Chemical reprogramming using small molecules offers unprecedented tools to produce footprint-free pluripotent cells without the use of exogenous transgene. In addition, small molecules used for reprogramming are cell permeable, non-immunogenic, easily synthesized and screened, protocols better standardized, and their effects on inhibiting and activating specific pathways are often reversible and can be finely adjusted (29).

Cell therapy aims at replacing damaged cells by healthy ones to restore function of organs and tissues, stimulating endogenous cells to promote tissue repair, or producing growth factors to stimulate the maturation of endogenous progenitors. Alternatively, donor cells can modulate the endogenous immune system to induce tolerance. The number of donor cells

available and the instability of the cellular phenotype during ex vivo expansion greatly limit the treatment of genetic diseases or pathologies, with prenatal and neonatal onset. Generating and banking pluripotent stem cells from fetal tissues can pave the way to use young rejuvenated cells to treat the fetus and prevent organ injury before irreversible damage in a fetal-to-fetal approach using cells from a similar developmental stage. There is the necessity to establish a GMP (Good Manufacturing Practice) compliant collection of human fetal stem cells for therapeutic grade banking (68). Although experimental research is at present at a preliminary preclinical stage and that proof of concept in larger animal models might be necessary before entering clinical trials; our strategy consists in developing GMP compliant cells during the lifetime of experimental research to allow rapid transition from bench to bedside when experimental data will support applications for clinical trials (69).

Because pluripotent stem cells form teratomas *in vivo*, they must be differentiated into specific lineages of interest *in vitro* and selected for absence of pluripotency marker expression (for example by absence of CD24 expression using flow cytometry cell sorting) prior to being injected. However, the capacity of iPS cells to differentiate is regulated by the Germ Cell Nuclear Factor (GCNF) (Nuclear receptor subfamily 6 group A member 1, NR6a1), a silencer of pluripotency genes required for the repression of pluripotency genes during development and used as a marker to distinguish completely reprogrammed iPS cells from incompletely pluripotent cells (70). Therefore, one of the challenges of using iPS cells for cell therapy is the ability to

completely repress the pluripotency network upon differentiation. For example, if OCT4 expression is maintained after differentiation, this leads to poor differentiation and aberrant expression of differentiated genes. In addition, the capacity of iPS cells to differentiate into specific lineage greatly depends on the tissue of origin, because of the epigenetic memory of the cells, which retain a propensity to differentiate into lineages of the parental cell population. While certain protocols are robust and well established, a number of them remain long and challenging.

Summary

Generation of induced pluripotent stem (iPS) cells can be achieved from various fetal and adult somatic cells and stem cells using different systems for the delivery of ectopic transcription factors, or by manipulation of the culture conditions and supplementation of the culture medium with small molecules to specifically inhibit or activate pathways involved in the establishment and maintenance of pluripotency. Whilst iPS cells can be generated from different tissues, the derivation of pluripotent stem cells from fetal tissues, in particular amniotic fluid, has important potential to treat a number of genetic disease or acute pathologies before birth or during the neonatal period. In addition, a chemical approach to reprogramming fetal tissues in mouse and humans allows the derivation of pluripotent cells free from exogenous DNA, without the risk of re-activation of reprogramming factors. Fetal stem cells have a lower incidence of genomic mutations, longer telomeres and active

telomerase, thereby producing younger iPS cells upon reprogramming. However, fetal tissues are not easy to isolate during ongoing pregnancy, with the exception of amniotic fluid, which is routinely sampled at mid-gestation for prenatal diagnostic.

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Conflict of interest statement:

None

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