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CICLO XXV

TITOLO TESI

Isolation and characterisation of mouse amniotic fluid stem cells: study of their origin, regenerative potential and reprogramming into pluripotent cells.

Direttore della Scuola: Ch.mo Prof. Giuseppe Basso

Coordinatore d'indirizzo: Ch.mo Prof. Giovanni Franco Zanon

Supervisore: Dott. Paolo De Coppi – Dott.ssa Michela Pozzobon

Dottorando: Enrica Bertin

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Most used abbreviations

AF: amniotic fluid

AFS: amniotic fluid stem

BM: bone marrow

BMP: bone morphogenetic protein

ES: embryonic stem

EG: embryonic germ

FGF: fibroblast growth factor

GFP: green fluorescence protein

GR: genital ridge

HSA: human α-skeletal actin

iPS: induced pluripotent stem

ICM: inner cells mass

LIF: leukemya inhibitory factor

MEF: mouse embryonic fibroblast

PB: piggy bac

pt: post tranplantation

SCF: stem cell factor

SMA: spinal muscolar atrophy

TA: Tibialis Anteriour

TNAP: tissue non speficic alkaline phosphatase

VPA: valproic acid

WT: wild type

I. Riassunto

Introduzione: Le cellule staminali hanno la capacità di dare orgine ad una progenie di cellule mature mantendo la capacità di autorinnovamento. Possono essere distinte sulla base delle loro potenzialità in totipotenti, pluripotenti, multipotenti, oligopotenti e unipotenti. Possono anche essere distinte in cellule staminali embrionali, fetali e adulte. Le cellule staminali embrionali, ottenute dalla massa cellulare interna della blastocisti, sono pluripotenti. Nell'embrione le cellule primordiali germinali danno origine ai gameti, ed esprimono i marcatori di pluripotenza (Oct4, Nanog, Sox2), ma non sono pluripotenti. Esse possono essere riprogrammate *in vitro*, diventando così cellule germinali embrionali. Tra le cellule staminali fetali ci sono le cellule staminali del liquido amniotico (AFS). Queste cellule sono isolate dal liquido amniotico per la positività al marcatore c-kit e sono presenti sia nell'uomo che nel topo, anche se la loro origine embrionale non è nota. Le cellule AFS umane sono multipotenti *in vitro*; le cellule AFS umane e murine hanno uno specifico potenziale ematopoietico, *in vivo* e *in vitro*. Recentemente è stato dimostrato che le cellule AFS umane ottenute dal primo e dal secondo trimestre di gravidanza, possono essere riprogrammate *in vitro* in cellule pluripotenti, a seguito dell'aggiunta di acido valproico. Inoltre, le cellule del liquido amniotico sono state anche utilizzate da diversi gruppi di ricerca per ottenere cellule staminali indotte alla pluripotenza (iPS). Per questi motivi, le AFS rappresentano una sorgente innovativa di cellule per approcci di medicina rigenerativa. L'atrofia spinale muscolare (SMA) è una malattia autosomica recessiva, causata della delezione o mutazione omozigote del gene della sopravvivvenza del motoneurone 1 (SMN1). Il trapianto di midollo osseo in un modello murino di SMA attenua il fenotipo miopatico, tuttavia non lo recupera totalmente e non mostra alcun effetto benefico a lungo termine.

Scopo della tesi: Gli scopi di questa tesi consistono nella caratterizzazzione delle cellule murine AFS isolate a fresco, nella valutaione del loro potenziale miogenico dopo il trapianto in animali HSA-Cre, $Smn^{F7/F7}$, nello studio della loro origine embrionale e nell'induzione alla pluripotenza usando un metodo non virale (PiggyBac, PB).

Materiali e Metodi: Le cellule murine AFS sono state ottenute attraverso amniocentesi e successiva immunoselezione per il marcatore c-kit mediante biglie magnetiche. Le cellule AFS sono state analizzate per l'espressione di diversi marcatori (CD90, CD45, CD44, CD34, CD31, Flk1, Sca1, CD105) attraverso la citometria a flusso e per l'espressione di Oct4, Sox2, c-Myc, Klf4, Nanog e Sca-1, attraverso qRT-PCR , a diversi stadi embrionali. Sono stati valutati il potenziale ematopoietico *in vitro* e la capacità di formare teratomi in topi $Rag2^{-/-}\gamma c^{-/-}$. Per la riprogrammazione a cellule embrionali germinali, le cellule AFS sono state seminate su *feeder layer* di STO o Sl^{4}-m220, mitoticamente inattivato, con il terreno per le cellule primordiali germinali, supplementato con LIF e il fattore per la crescita dei fibroblasti (bFGF) e successivamente con il terreno N2B27 2iLIF. Per il trattamento dei topi HSA-Cre, $Smn^{F7/F7}$ le cellule AFS isolate da topi GFP^{+}, sia isolate a fresco che coltivate, sono state iniettate nelle vena della coda e i topi sono stati sacrificati un mese dopo trapianto ed i muscoli analizzati mediante ematossilina ed eosina, tricromica di Masson e con immunofluorescenza per l'espressione di distrofina/GFP. Per studiare l'origine embrionale delle cellule AFS sono stati utilizzati due modelli murini: Oct4-GFP e TNAP-Cre. Per l'induzione alla pluripotenza, cellule ottenute dai feti Oct4-GFP son state trasfettate con i plasmidi del trasposone PB-TET contenente quattro geni (Oct4, Sox2, c-Myc e Klf4) sotto il controllo trascrizionale del promotore inducibile $tetO_2$ tetraciclina/ doxiciclina. L'espressione dei geni della pluripotenta è stata indotta con la doxiciclina. Le cellule iPS così ottenute sono state testate per l'espressione dei marcatori Nanog, SSEA-1 e per la positività alla fosfatasi alcalina.

Risultati: Le cellule AFS $c\text{-}kit^{+}$ murine variano in numero durante il corso della gestazione. Queste cellule esprimono i marcatori ematopoietici (CD45, CD34, Sca1), mesenchimali (CD90, CD105) insieme a Flk1, CD31, CD44. Sulla base dell'epressione di c-kit sono state identificate due popolazione cellulari: $c\text{-}kit^{high}$ e $c\text{-}kit^{low}$, che mostrano anche una differente espressione dei marcatori sopracitati. Le cellule $c\text{-}kit^{low}$ sono presenti in numero maggiore e durante il corso della gestazione diminuiscono mentre le $c\text{-}kit^{high}$ aumentano. Entrambe le popolazioni hanno un potenziale ematopoieitco *in vitro*. Le cellule murine AFS esprimono a bassi livelli i geni Oct4 e Sox2 e ad alti livelli c-Myc e Klf4, ma la loro espressione cambia durante il corso della gestazione. La stessa analisi eseguita a livello di singola cellula ha mostrato che

allo stadio E13.5 il 5% delle cellule co-esprime Oct4, Sox2 e Klf4. Le cellule AFS murine non formano teratoma. Negli esperimenti di terapia cellulare topi HSA-Cre, $Smn^{F7/F7}$ non trattati, morivano all'età di 10 mesi mentre topi trattati con le cellule AFS o cellule da midollo osseo non frazionato avevano una sopravvivvenza del 75% e del 50%, rispettivamente. I topi trattati con le cellule AFS, mostravano un recuperano della forza muscolare rispetto agli animali non trattati (+75%). I muscoli degli animali trattati con le cellule AFS mostravano una morfologia normale con un numero basso di fibre rigeneranti (<1%) e una normale espressione delle distrofina che per il 37.86% (± 9.48%) era GFP^+. A 15 mesi dal trattamento, gli animali trattati con cellule del midollo osseo avevano un maggior numero di fibre centro nucleate e una consistente infiltrazione di tessuto interstiziale e nessuna fibra GFP^+. Gli animali trattati con le cellule AFS mostravano un fenotipo migliore e il 58.00% (± 2.43%) delle fibre muscolari era GFP^+. Risultati simili sono stati ottenuti trattando topi HSA-Cre, $Smn^{F7/F7}$ con cellule AFS espanse *in vitro*.

Per cercare di differenziare le cellule AFS in cellule embrionali germinali pluripotenti abbiamo testato due protocolli ma nessuna colonia che ricordasse cellule embrionali germinali si è formata *in vitro*. Le cellule AFS isolate dai feti Oct4-GFP erano GFP negative. Il modello TNAP-Cre è risultato essere aspecifico, perchè TNAP non risultava essere espresso solo nelle cellule germinali primordiali. Negli esperimenti di riprogrammazione, alcuni cloni di iPS hanno mostrato di essere doxiciclina indipendenti, esprimevano il gene Oct4 endogeno ed erano positivi per i marcatori Nanog e SSEA1 e per la fosfatasi alcalina.

Discussione: Le cellule murine AFS sono una popolazione eterogenea, le cui caratteristiche variano durante il corso della gestazione ed esprimono marcatori ematopoietici, mesenchimali e anche marcatori tipici delle cellule endoteliali. Il potenziale differenziativo delle due popolazioni c-kit^{high} e c-kit^{low} dovrà essere testato *in vivo*. L'analisi dell'espressione genica a livello di popolazione e a livello di singola cellula per i marcatori della pluripotenza ha confermato l'eterogeneità delle cellule AFS. Il trattamento degli animali HSA-Cre, $Smn^{F7/F7}$ ha mostrato un potenziale miogenico delle cellule murine AFS, anche a lungo termine, suggerendo un interessante potenziale terapeutico. Le cellule AFS potrebbero contribuire a generare nuove fibre mus-

colari fondendosi con fibre esistenti o integrarsi nelle nicchia delle cellule staminali muscolari, ma maggiori esperimenti saranno necessari per valutare la validità dell'ipotesi. Anche le cellue AFS coltivate hanno dimostrato il mantenimento delle proprietà rigenerative. Questo studio suggerisce che le cellule AFS murine probabilmente non derivano da cellule primordiali germinali. Tuttavia è importante ricordare che il modello murino Oct4-GFP non è un modello di lineage tracking. Maggiori esperimenti saranno necessari per confermare questi risultati e per identificare l'origine delle cellule AFS. Le cellule iPS sono un promettente strumento di ricerca come modello di malattia o nella speranza di ottenere una sorgente di cellule per terapia. Qui è stato dimostrato come il sistema del PB può essere un valido metodo per la riprogrammazione delle cellule murine AFS. Questi sono solo risultati preliminari e maggiori esperimenti saranno necessari per completarne la caratterizzazione.

II. Abstract

Introduction: Stem cells are defined by their ability to proliferate for a long period of time, a property known as 'self-renewal', and to give rise to differentiated cells. Stem cells can be distinguished into totipotent, pluripotent, multipotent, oligopotent and unipotent. They can also be classified into embryonic, adult and fetal stem cells. Embryonic stem (ES) cells are obtained from inner cell mass (ICM) of blastocyst and are puripotent. Primordial germ cells (PGC) in the embryo give rise to gametes but they are not pluripotent, albeit they express Oct4, Nanog and Sox2. They can be reprogrammed *in vitro*, becoming pluripotent embryonic germ (EG) cells. Amniotic fluid stem (AFS) cells are fetal stem cells that can be isolated from the amniotic fluid (AF) by the expression of the marker c-kit, both in human and mouse, but their origin is unknown. Human AFS are multipotent *in vitro*, while both human and mouse AFS have hematopoitic potential, *in vitro* and *in vivo*. Recently it has been demonstrated that human AFS from first and second thrimester can be reprogrammend into pluripotent cells *in vitro*, after supplementation with Valproic Acid (VPA). Cells from the AF have also been used to obtain induced pluripotent stem (iPS) cells. For all these reasons AFS cells seems to be a promising sources of cells for regenerative medicine. Spinal muscular atrophy (SMA) is an autosomal recessive disease, caused by an homozygous deletion or mutation of the motor neuron 1 (SMN1) gene. Bone marrow (BM) transplantation in a murine model of SMA attenuates the myopathic phenotype without a full recovery and without long-term therapeutic effects.

Aims of the thesis: Characterisation of fresh mouse AFS cells, evaluation of their myogenic potential into a model of SMA (HSA-Cre, $Smn^{F7/F7}$ mouse) investigation of their putative PGC origin and induction to pluripotency through a non-viral method (PiggyBac, PB).

Materials and Methods: Mouse AFS cells were obtained by amniocentesis and selected as c-kit^{+} cells with magnetic beads. Freshly isolated-AFS cells were analized for the expression of different markers (CD90, CD45, CD44, CD34, CD31, Flk1, Sca1, CD105) by flow citometry and the expression of Oct4, Sox2, c-Myc, Klf4 and

Sca-1 by qRT-PCR at different embryonic stages. Hematopoitic potential was evaluated *in vitro*, while the teratoma assay was performed in $Rag2^{-/-}\gamma c^{-/-}$ mice. For the reprogramming into EG cells cells were seeded into a feeder layer of mitotically inactivated STO or Sl^{4}-m220, in a PGC medium supplemented with LIF and basic fibroblast growth factor (bFGF) and in N2B27 2iLIF medium. For the treatment of HSA-Cre, $Smn^{F7/F7}$ mice, GFP^{+} cells were injected via the tail vain and sacrificed one month after transplantation. *Tibialis Anteriour* (TA) muscles were stained with hematoxylin and eosin, Masson's trichrome and analized by immunofluorescence for positivity for GFP/dystrophin. The experiments for the origin of AFS have been conducted using two mouse models: Oct4-GFP and TNAP-Cre. For the induction to pluripotency cells were obtanied from Oct4-GFP positive embryos, and transfected with the PB-TET transposon plasmid containing four genes (Oct-4, Sox-2, c-Myc and Klf4) under the transcriptional control of the $tetO_2$ tetracycline/doxycycline inducible promoter. The expression of pluripotency gene was induced with doxycycline. iPS cells obtained were tested for the expression of Nanog, SSEA-1 and for positivity to alkaline phosphatase.

Results: Mouse AFS number chaged during the course of gestation. These cells expressed hematopoietic markers (CD45, CD34, Sca1), mesenchymal markers (CD90, CD105) together with Flk1, CD31 and CD44. On the basis of c-kit expression two populations were defined: $c\text{-}kit^{high}$ and $c\text{-}kit^{low}$ which showed differential expression of the aforementioned markers. $c\text{-}kit^{low}$ are the more abundant, but during the course of gestation they decreases in numbers while the number of $c\text{-}kit^{high}$ cells increases. Both populations had hematopoietic potential *vitro*. Gene expression analysis showed that mouse AFS cells expressed at low levels Oct4 and Sox2 and high levels c-Myc and Klf4, and their expression changed during the course of gestation. Single cell PCR showed that at E13.5 there 5% of cells co-expressed Oct4, Sox2 and Klf4. Mouse AFS cells didn't form teratoma. In the cell therapy experiments HSA-Cre, $Smn^{F7/F7}$ control mice died at the age of 10 months, while mice treated with GFP^{+} AFS or bone marrow (BM) cells had a survival rates increased by 75% and 50% respectively. HSA-Cre, $Smn^{F7/F7}$ mice treated with AFS cells recovered more than 75% of force compared to the untreated animals. One month after transplantation, muscles from AFS-treated mice displayed very low number of regenerating myofibers

(<1%) and normal dystrophin expression; moreover, 37.86% (± 9.48%) of the fibers were GFP^+. 15 months after transplantation BM-treated mice displayed a high number of central nucleated fibers and consistent infiltration of interstitial tissue and no GFP^+ myofibers, while AFS-treated mice had a mild-phenotype, close to wild-type mice, and 58.00% (± 2.43%) of the myofibers were GFP^+. Similar results were obtained with HSA-Cre, $Smn^{F7/F7}$ treated with mouse AFS cells expanded in culture.

To evaluate if mouse AFS cells were PGC cells, they were cultivated following the protocol established to obtain pluripotent EG cells from PGC cells. Two different culture protocols were used, but no EG cells were obtained. AFS cells isolated from Oct4-GFP fetuses at different embryonic stages showed no presence of $Oct4^+$ cells. The TNAP-Cre line resulted to be unspecific. iPS clones obtained transfecting mouse AFS cells were doxycycline indipendent, they expressed Oct4, they were positive for Nanog and SSEA1, and for the alkaline phosphatase.

Discussion: Mouse AFS cells are an heterogenous population, and their phenotype changed during the course of gestation. They expressed mesenchymal, hematopietic and endothelial markers. The two populations ($c\text{-}kit^{high}$ and $c\text{-}kit^{low}$) should be tested *in vivo* to asses their differentiative potential. Gene expression analysis at population and single cells level confirmed the heterogeinity of mouse AFS cells. AFS showed a myogenic potential, even after long-term transplantion, suggesting an interesting therapeutic potential of these cells. AFS could contribute to the formation of new myofibers by fusing with existing ones or after integration within the stem cell niche of the muscle. The study of their origin suggested that mouse AFS cells aren't PGC. However it is important to remind that the Oct4-GFP mouse is not a lineage-tracking model; therefore more experiments are needed to confirm these results and to find the origin of these cells. iPS cells are a promising research tool to obtain a model of several diseases or as a source of cells for therapeutic approaches. Here it has been shown that the PB system is a suitable method for the reprogramming of mouse AFS cells. These are only preliminary results and more experiments will be necessary to complete the characterisation of these cells.

III. Introduction

1. Stem cells

Stem cells are cells present in all multicellular organisms and maintain tissue homeostasis by replacing terminally differentiated, aged or injured cells. Stem cells are defined by their ability to proliferate for a long period of time, a property known as 'self-renewal', and to give rise to differentiated cells. Typically, stem cells generate intermediate cell types, called precursor/progenitor both in adult e fetal tissues, before they achieve a fully differentiated state [1]. Stem cells can be divided in different groups on the basis of their potency or plasticity: 1) Totipotent stem cells: able to constitute an entire organism. (i.e. the zygote, from which the embryo and extraembryonic structures origin); 2) Pluripotent stem cells: able to form all the body's cell lineages, including germ cells .(i.e. embryonic stem cells); 3) Multipotent stem cells: form multiple cell lineages that constitute an entire tissue. (i.e.: haematopoietic stem cells); 4) Oligopotent stem cells: able to form two or more lineages within a tissue. (i.e:. neural stem cells can create a subset of different neurons in the brain); 5) Unipotent stem cells: form a single lineage. (i.e.: spermatogonial stem cells).

Stem cells can be also classified on the basis of their origin in a) embryonic, b) adult and c) fetal stem cells.

(a) Embryonic Stem Cells

To date stem cell lines that have been derived from the embryo are the i) embryonic stem (ES) cells [2,3] ii) embryonic germ (EG) cells [4] and epiblast stem cells (EpiSC) [5,6]. (Figure 1)

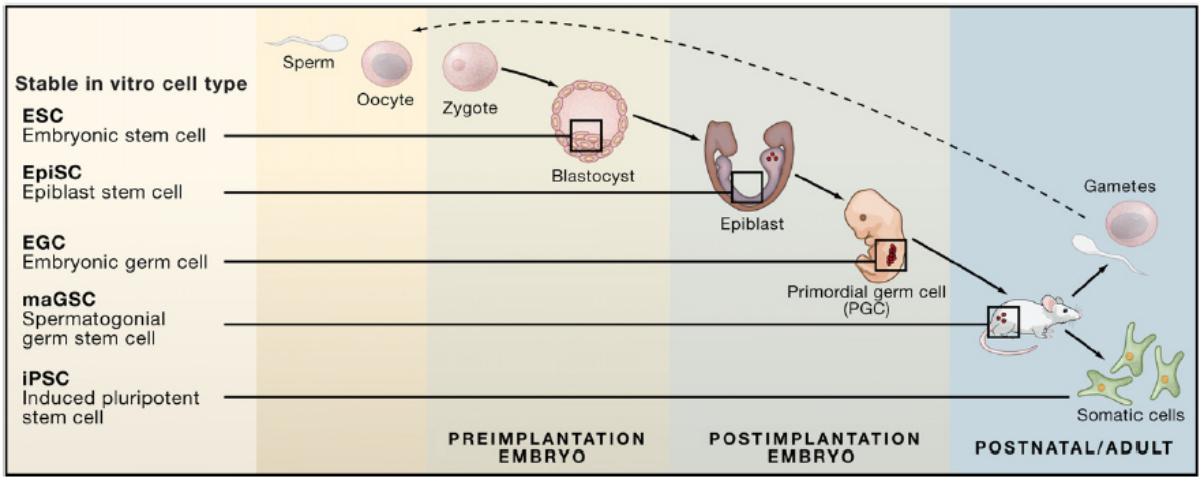


Figure 1: **Developmental origins of pluripotent stem Cells.** Different types of pluripotent cells can be derived by explanting cells at various stages of early embryonic development. Modified from [7]

(i) ES cells

The first ES cells were successfully derived directly from mouse blastocysts in 1981; when inner cells mass (ICM) cells were put in particular culture condition, they proliferated in the form of mouse ES cells [2,3]. These cells are pluripotent, in fact when injected into a host blastocyst they are able to repopulate an embryo (forming chimera embryo) and to contribute descendents to all tissues of the adult, including the germline [8,9]. Mouse ES cells can be maintained *in vitro* using a medium containing fetal bovine serum (FBS) and leukaemia inhibitory factor (LIF), with the addition of a mouse embryonic fibroblast (MEF) feeder layer [2]. Recently, it has been shown that mouse ES cells can self-renew in basal medium if autocrine mitogen-activated protein kinase (MAPK) signalling is blunted and glycogen synthase kinase 3 (GSK-3) activity is reduced [10]. The two inhibitor (2i)-culture system, supplemented with LIF, allows efficient derivation and expansion of germline-competent ES cells from different strains of mice, including 129, CBA and NOD [9,10], and, for the first time, from the rat [11,12]. Markers that are used to characterized mouse ES cells are: Alkaline phosphatase [13], Oct4 [14], Sox2 [15], Nanog [16,17] and SSEA1 [18].

(ii) EG cells and Primordial Germ cells

The generation of mouse embryonal carcinoma (EC) cells from teratocarcinomas that arose spontaneously in male gonads [19] prompted speculation that it may be possible to derive pluripotent stem cell lines directly from primordial germ cells (PGC). In the embryo PGC are cells that gave rise to gametes. During mouse embryo development germ cells undergo through a series of changes, that bring them to maruration. Different phases are distinguishable (Figure 2): 1) Specification: Specification of PGC is initiated through signals provided by the extraembryonic ectoderm (ExE), and the visceral endoderm (VE) that surrounds the epiblast cells and instructs a small number of epiblast cells to become PGCs [20]. Both the ExE and VE are the sources of bone morphogenetic protein (BMP) 4, 8b, and 2 which are required for the acquisition of competence in PGC precursors [21]. Specification start around E5.5 and it finished at E8.0, during this phase PGCs express different markers, Blimp-1, Fragilis, Stella, Nanos3, Prdm14 plus mesodermal genes including T, Fgf8, and Snail become repressed, whereas pluripotency-associated genes such as Sox2, Oct4, Nanog are upregulated. PGCs are also recognizable for the activity of the alkaline phosphatase [22,23]. After specification, the germ cells become transcriptionally silent at E8.5 and are subjected to an extensive reprogramming of their genomes.[24] 2) Migration: From around E7.5, PGCs start to migrate to the hindgut, then toward the mesentery and finally they reach genital ridges (GRs), which they colonize by E10.5 to initiate sexually dimorphic development [23] (Figure 2). During their migration and early period in the GRs, PGCs undergo active proliferation [25]. However, at E13.5, both male and female germ-cell growth is arrested, and female germ cells immediately enter prophase of the first meiotic division [26], while male germ cells are arrested and enter the G0 phase of the cell cycle until birth, when prospermatogonia resume mitosis [27].

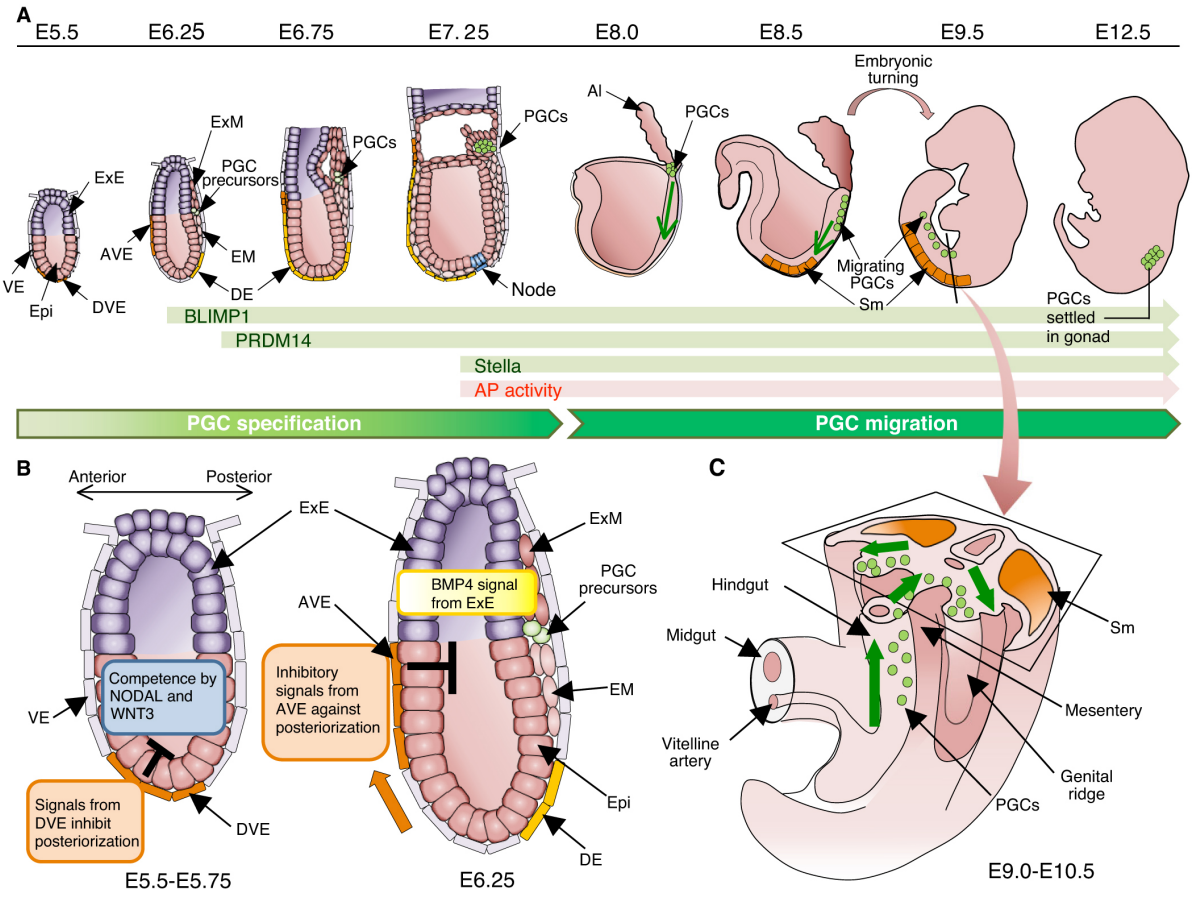


Figure 2: **Specification and migration of mouse primordial germ cells.** (A) A schematic of germ cell specification and migration in developing mouse embryos. PGC precursors (E6.25) and PGCs are shown as green circles in embryos from E6.25 to E12.5, and the direction of migration is denoted by a green arrow. The timing of expression of key genes (Blimp1, Prdm14 and Stella) and alkaline-phosphastase activity is shown below. (B) Signalling activities for PGC specification at E5.5-E5.75 and at E6.25. (C) A view of PGC migration from the hindgut through the mesentery to genital ridges at E9.0-E10.5. The direction of PGC migration is denoted by a green arrow and anterior is towards the top. Al, allantois; AVE, anterior visceral endoderm; DE, distal endoderm; DVE, distal visceral endoderm; EM, embryonic mesoderm; Epi, epiblast; ExE, extra-embryonic ectoderm; ExM, extra-embryonic mesoderm; PGCs, primordial germ cells; Sm, somite; VE, visceral endoderm. Modified from [23].

Despite the expression of the pluripotent stem cells markers (like Oct4, Nanog and Sox2) PGCs are unipotent, they can differentiate only into sperm or eggs, and if injected into an host blastocyst, they don't contribute to chimeras [28,29]. Pluripotency is achieved by culturing the posterior region of E8.5 mouse embryos, or GRs at E10.5 on feeder cells in the presence of particular cytokines, like LIF, steel factor/ stem cells factor (SCF), basic fibroblast growth factor (bFGF) and serum [4]. Pluripotent cell obtained with this method are called EG cells and they closely resemble ES

cells [30]. FGF appears not to be necessary if PGCs are cultured directly in 2i medium with LIF [31]. EG cells can be obtained also from explanted GRs at E11.5 and E12.5, but by this stage, the developing germ cells have undergone imprinting erasure, which renders the derivative EG cells less able to integrate into chimaeras, and particularly into the germline. [32]. Derivation of human cells with features of EG cells has also been reported [33,34]. However, although these cultures express pluripotency marker genes and demonstrate broad differentiation, undifferentiated cells have not been clonally propagated or maintained during long-term culture yet [35]. Thus, whether the ability to reprogram to pluripotent stem cells *in vitro* is a phenomenon specific to mice or is a general property of mammalian PGCs remains an open question.[31]

(b) Adult Stem Cells

Adult stem cells are capable of maintaining, generating, and replacing terminally differentiated cells within their own specific tissue as a consequence of physiologic cell turnover or tissue damage due to injury. Adult stem cells are rare: within the bone marrow (BM), for example, truly hematopoietic stem cell (HSC) are less than the 0.01% [36]. Furthermore, adult stem cells are dispersed in tissues and behave very differently, depending on their environment. For example, HSCs are constantly renewed in the bone marrow where they differentiate into mature types of blood cells [37]. On the contrary stem cells in the small intestine are stationary, and are physically separated from the mature cell types they generate [38]. Adult stem cells can be found in different tissues like brain, dental pulp, muscle, bone marrow, skin, intestine and pancreas and have been extensively characterized also for a therapeutic exploitation. Different studies have shown that blood stem cells (derived from mesoderm) may be able to generate both skeletal muscle (also derived from mesoderm) [39] and neurons (derived from ectoderm) [40], however the “plasticity” of adult somatic stem cells is still controversial, and in general, existing evidence suggests that *in vivo* such unexpected transformations are exceedingly rare.[41]

(c) Fetal Stem Cells

Fetal stem cells (FSCs) can be found in fetal tissues such as blood, liver, bone marrow, pancreas, spleen and kidney, and stem cells are also found in cord blood and extraembryonic tissues such as amniotic fluid, placenta and amnion. Their primitive properties, expansion potential and lack of tumorogenicity make them an attractive option for regenerative medicine in cell therapy and tissue engineering approaches. [42]

(i) Amniotic Fluid Stem Cells

In humans, the AF generates at the beginning of the second week of gestation as a small film of liquid between the cells of the epiblast [43]. The fluid expands separating the epiblast (the future embryo) from the amnioblasts (the future amnion), thus forming the amniotic cavity [44]. The origin of amniotic fluid cells is still debated [45,46] but, it is known that the majority of cells within the amniotic fluid are terminally differentiated and have limited proliferative capacity [47,48]. Different studies have demonstrated the presence of a subset of cells with a proliferative and differentiation potential also in the AF [49], these include cells found in mid-gestation expressing the haematopoietic marker CD34 [50] as well as cells with mesenchymal features (MSCs), able to proliferate *in vitro* more rapidly than mesenchymal fetal and adult cells [51,52,53,54,55,56,57]. Amniotic fluid MSCs are negative for hematopoietic markers such as CD45, CD34 and CD14 [54,55,56,57,58]. Despite their high proliferation rate, these cells display a normal karyotype when expanded *in vitro* and do not form tumours *in vivo* [52]. They exhibit a broad differentiation potential towards mesenchymal lineages [54,55,59,60,61]. De Coppi et al. isolated c-kit-positive (CD117) cells that represent about 1% of cells present in second-trimester amniotic fluid. These cells were named amniotic fluid stem (AFS) cells. They can be cultured without feeders, double in 36h, are not tumorigenic, have long telomeres and retain a normal karyotype for over 250 population doublings [62]. Cultured human AFS cells

are positive for ES cell (e.g. Oct-4, Nanog and SSEA-4) and mesenchymal cell markers such as CD90, CD105 (SH2), CD73 (SH3/4) and several adhesion molecules (e.g. CD29 and CD44) [62,63]. Furthermore, it was possible to generate clonal human lines from these cells, verified by retroviral marking, which were capable of differentiating into lineages representative of all three embryonic germ layers. Almost all clonal AFS cell lines express Oct-4 and Nanog, markers of a pluripotency [62]. c-kit$^+$ lineage$^-$ cells from human and mouse amniotic fluid display a multi-lineage haematopoietic potential *in vitro* and *in vivo* [64]. The potentiality of AFS cells have been tested in various models of diseases, it has been demostrated that they contribute to lung [65], kidney [66], cardiac [67], bone [68], and smooth muscle regeneration [69,70]. Moschidou et al. showed that human c-kit$^+$ cells of first and mid-trimester can be fully reprogrammed to pluripotency by culture on Matrigel in human embryonic stem (hES) cell medium supplemented with the histone deacetylase inhibitor (HDACi) valproic acid (VPA). The reprogrammed cells share 82% transcriptome identity with hES cells and are capable of forming embryod bodies (EBs) *in vitro* and teratomas *in vivo*. After long-term expansion, they maintain genetic stability, protein level expression of key pluripotency factors, high cell-division kinetics, telomerase activity, repression of X-inactivation, and capacity to differentiate into lineages of the three germ layers, such as definitive endoderm, hepatocytes, bone, fat, cartilage, neurons, and oligodendrocytes. [71,72]

2. Induced Pluripotent Stem Cells

In 2006 Takashashi and Yamanaka found that the ectopic expression of defined factors into a somatic cell could reprogram differentiated cell into a pluripotent cell, that was called induced pluripotent stem (iPS) cell. They screened a pool of 24 pluripotency-associated candidate genes, coexpressed with retroviral vectors in mouse fibroblasts which had a dormant drug resistance allele integrated into the ESC-specific Fbxo15 locus. The combination of 24 factors activated Fbxo15 and induced the formation of drug-resistant colonies with characteristic ES cells morphology [73]. Successive rounds of elimination of individual factors then led to the

identification of the minimally required core set of four genes: Klf4, Sox2, c-Myc, and Oct4. iPSCs generated by selection for Fbxo15 activation expressed markers of pluripotent stem cells such as SSEA-1 and Nanog, generated teratomas when injected subcutaneously into immunocompromised mice, and contributed to different tissues of developing embryos when injected into blastocyst [73]. However, these ‘‘first-generation’’ iPSCs were only partially reprogrammed, in fact they expressed lower levels of several key pluripotency genes compared with ES cells, showed incomplete promoter demethylation of ES cell regulators such as Oct4, and failed to generate postnatal chimeras or contribute to the germline. Different laboratories improved the iPS cells generated for example using the selection for Nanog or Oct4 instead of Fbxo15 [74,75,76]. Recently, rare iPSC lines have been identified that are even capable of generating ‘‘all-iPSC’’ mice upon injection into tetraploid blastocysts [77,78][79,80]. iPSCs have also been derived from a number of different species, including humans [81,82,83], rats [84], and rhesus monkeys [85] and from other somatic cells beyod fibroblasts, such as keratinocytes [86,87], neural cells [88,89], stomach and liver cells [90], and melanocytes [91], as well as from genetically labeled pancreatic β cells [92] and terminally differentiated lymphocytes [88,93]. Different groups have also used amniotic fluid cells as source for obtain iPS cells. These works are summarized in the table below.

CellIs	Factors	Method of delivery	References
human and mouse amniotic fluid derived cells (the last one selected for c-kit and not selected)	Oct4, Sox2, c-Myc, klf4 (also only klf4 and c-Myc for mouse cells)	retroviral vector	[94]
human amniotic fluid derived cells from β-thalassemia patient	Oct4, Sox2, c-Myc, Klf4	lentiviral vector (doxycycline-inducible)	[95]
human amniotic fluid-derived cells	Oct4, Sox2, c-Myc, Klf4	retroviral vector	[96]
human amniotic fluid-derived cells	Oct4, Klf4	retroviral vector	[97]
human amniotic fluid derived cells from trisomy 21 patient	Oct4, Sox2, c-Myc, Klf4	lentiviral vector	[98]
human amniotic fluid-derived cells	Oct4, Sox2, c-Myc, Klf4	retroviral vector	[99]
human amniotic fluid derived cells from β-thalassemia patient	Oct4 and Sox2 or Oct4, Sox2, and Klf4	retroviral vector	[100]
human amniotic fluid-derived cells	Oct4, Sox2, c-Myc, Klf4	bacteriophage ΦC31 integrase	[101]
human amniotic fluid-derived cells CD34+	Oct4	retroviral vector	[102]
human amniotic fluid-derived cells	Oct4, Sox2, c-Myc, Klf4	retroviral vector	[103]
human amniotic fluid-derived cells	Oct4, Sox2, c-Myc, Klf4	retroviral vector	[104]

Table 1: **iPS obtained from human/mouse amniotic fluid stem cells.**

(a) Factor delivery into target cells

(i) Viral delivery methods

In 2006 Takahashi et al., used constitutively active retroviral vectors that stably integrated into the host cell genome to introduce c-Myc, Klf4, Oct4, and Sox2 into the somatic cells [105]. Even if retroviral transgenes are usually silenced toward the end of reprogramming [106], this process is often incomplete, resulting in partially reprogrammed cell lines that continue to depend on exogenous factor expression and fail to activate the corresponding endogenous genes [73,107,108]. In addition, residual activity or reactivation of viral transgenes in iPS cell-derived somatic cells can interfere with their developmental potential [73] and frequently leads to the formation of tumors in chimeric animals [75]. Lentiviral vectors are also used to produce iPS cells, but they are are even less efficiently silenced in pluripotent cells than retroviral vectors [109,110]. The use of inducible lentiviral vectors, whose expression can be controlled by the inert drug doxycycline, diminishes the risk of continued transgene expression and allows for the selection of fully reprogrammed iPS cells, since cells that depend on exogenous factor expression readily stop proliferating upon doxycycline withdrawal [106,109]. Lentiviral vectors are also more efficient than retroviral vectors at infecting different somatic cell types and can be used to express polycistronic cassettes encoding all four reprogramming factors, thus increasing reprogramming efficiency [110,111]. To summarize the integrating methods to reprogramm somatic cells into iPS cells have adavantages to have the highest efficiency and in the case of lentiviral vector, to transduce dividing and non dividing cells, on the other hand they integrate into the genoma of cells, with an incomplete proviral silencing and with the risk of insertional mutagenesis and tumor incidence.

(ii) Integration-free iPSCs

Approaches to derive iPS cells free of transgenic sequences are aimed at circumventing the potentially harmful effects of leaky transgene expression and insertional mutagenesis. This is relevant when considering iPS cells technology in a therapeutic setting. Techniques to generate integration-free iPS cells can be subdivided into

three categories: (1) those that use vectors that do not integrate into the host cell genome, like for example adenoviruses and plasmids, (2) those that use integrating vectors that can be subsequently removed from the genome, like for examples transposons and loxP-flanked lentiviruses, and (3) those that do not use DNA-based vectors at all, such as Sendai virus, proteins, modified mRNA and microRNA. The first integration-free iPSCs were generated from adult mouse hepatocytes using nonintegrating adenoviral vectors [112], and from MEFs transfected with plasmids [75]. These experiments provided the proof of principle that transient expression of the four classical reprogramming factors is indeed sufficient to induce pluripotency in somatic cells. Human fibroblasts have also been reprogrammed into iPSCs with adenoviral vectors [113] and Sendai virus [114], as well as with polycistronic minicircle vectors [115] and self-replicating selectable episomes [116], albeit the latter system required the simultaneous overexpression of additional factors, including another potent oncogene (Oct4, Sox2, c-Myc, and Klf4, together with Nanog, Lin28, and SV40LT). Reprogramming efficiencies with current non-integrating methods are several orders of magnitude lower (0.001%) than those achieved with integrating vectors (0.1%–1%), most likely because factor expression is not maintained for a sufficient length of time to allow complete epigenetic remodeling. To avoid this issue, several laboratories have developed integration-dependent gene delivery vectors with incorporated loxP sites that can be subsequently excised from the host genome by transient expression of Cre recombinase [117,118]. This approach enables the efficient generation of iPSCs from different cell types, especially if polycistronic vectors are used [110,119]. Transgene-free iPSCs can also be generated with PB transposons, mobile genetic elements that can be introduced into and removed from the host genome by transient expression of transposase [120,121]. Successful reprogramming has been achieved recently without the use of viral or plasmid vectors at all. Specifically, iPSCs have been derived from both mouse and human fibroblasts by delivering the reprogramming factors as purified recombinant proteins [79] or as whole-cell extracts isolated from either ESCs [122] or genetically engineered HEK293 cells [123]. While the use of purified proteins represents an attractive approach for the generation of transgene-free iPSCs, its efficiency is extremely low and, in the recombinant protein approach, required the addition of VPA to the culture me-

dia. A more efficient and safer way of producing integration-free iPSCs may be the introduction of modified RNA molecules encoding for the reprogramming factors into somatic cells [124]. To improve the overall low efficiencies of generating iPSCs with most non-integrating approaches, screens for chemical compounds that promote reprogramming have been performed. This led to the identification of a number of molecules that significantly increase reprogramming efficiencies in the context of Oct4, Klf4, Sox2, and c-Myc overexpression [125,126]. Notably, some of these molecules can also replace individual reprogramming factors, raising the possiblility of deriving iPSCs solely with chemicals [125,126]. To summarize the integration-free methods to obtain iPS cells have the advantages to avoid the genomic integration, but they have a low efficiency.

(b) Identification of iPSC colonies

It is really important the selection of successfully reprogrammed clones from partially reprogrammed or simply transformed iPS colonies. The reactivation of endogenous pluripotency-associated genes such as Fbxo15 [73], Nanog or Oct4 [74,75,76], and Utf1 [127] linked to drug selection cassettes has been successfully employed for this purpose. A general limitation of any drug selection approach is that it requires genetic engineering of cells or mice. To circumvent this problem, lentiviral vector systems have been developed that carry promoter fragments of pluripotency genes whose activity can be selected for, and that, in principle, can be applied to a wide range of murine and human cell types [128]. For human iPS cells, expression of surface markers such as TRA-1-81 has been shown to enrich for reprogrammed cells [129]. A more stringent approach to identify faithfully reprogrammed human iPSCs without the use of drug selection combines the detection of surface markers with that of ‘‘indicator retroviruses’’ expressing fluorescent proteins, which become silenced upon acquisition of pluripotency [130]. Importantly, high-quality iPSCs can be derived from unmodified somatic cells without drug selection or fluorescent reporters at all by simply using morphological criteria [74,131,132], although this approach requires careful characterization of the resultant cell lines. This ‘‘no selection’’ approach is therefore most powerful when combined with doxycycline-inducible vectors, as cells that have

entered a self-sustaining pluripotent state can be easily selected by removal of doxycycline [106,109], even though, in rare cases, doxycycline-independent partially reprogrammed cells have been reported [107].

(c) Therapeutic potential of iPSCs

The generation of patient-specific stem cells has been a long-standing goal in the field of regenerative medicine. This point has been reached thank to discovery of iPS cells. iPS cells have a lot of potentiality in fact they provide a unique platform from which to gain mechanistic insight into a variety of diseases, to carry out *in vitro* drug screening, to evaluate potential therapeutics and to explore gene repair coupled with cell-replacement therapy (Figure 3). Experiments in mice suggest that the treatment of genetic disorders with iPS cells is feasible: Jaenisch and colleagues [133] showed that iPSCs can be used to rescue the defects seen in an animal model of sickle cell anemia, in another study mice transplanted with heterologous, iPSC-derived endothelial progenitor cells corrected a phenotype of hemophilia A [134]. The study and treatment of many degenerative diseases, such as type I diabetes, Alzheimer's disease, and Parkinson's disease, is limited by the accessibility of the affected tissues, as well as the inability to grow the relevant cell types in culture for extended periods of time. The idea behind so-called ''disease modeling'' is to derive iPSCs from patients' skin cells and then differentiate them *in vitro* into the desired cell types, thereby recapitulating the disease in a cell culture dish. The advantage of this approach over currently used strategies is that the very cell type that is compromised can be recreated in culture to be studied, even when the cell type is long gone from the patient. Moreover, because iPSCs grow indefinitely in culture, they provide an unlimited source for any desired specialized cells. Ultimately, the goal of this approach is to use create models of disease as a platform to identify novel drugs. Several laboratories have already derived iPSCs from patients suffering from Huntington's and Parkinson's disease, ALS, juvenile diabetes, muscular dystrophy, Fanconi anemia, Down syndrome, and others pathologies [118,135,136,137], moreover, three reports showed that iPSCs derived from patients suffering from the disorders SMA [138], familial dysautonomia (FD) [139], and LEOPARD syndrome [140] recapitulated cell ab-

normalities *in vitro* as seen in patients. Remarkably, when the cultured cells were exposed to experimental drugs for these diseases, the ‘‘symptoms’’ were partially alleviated in culture.

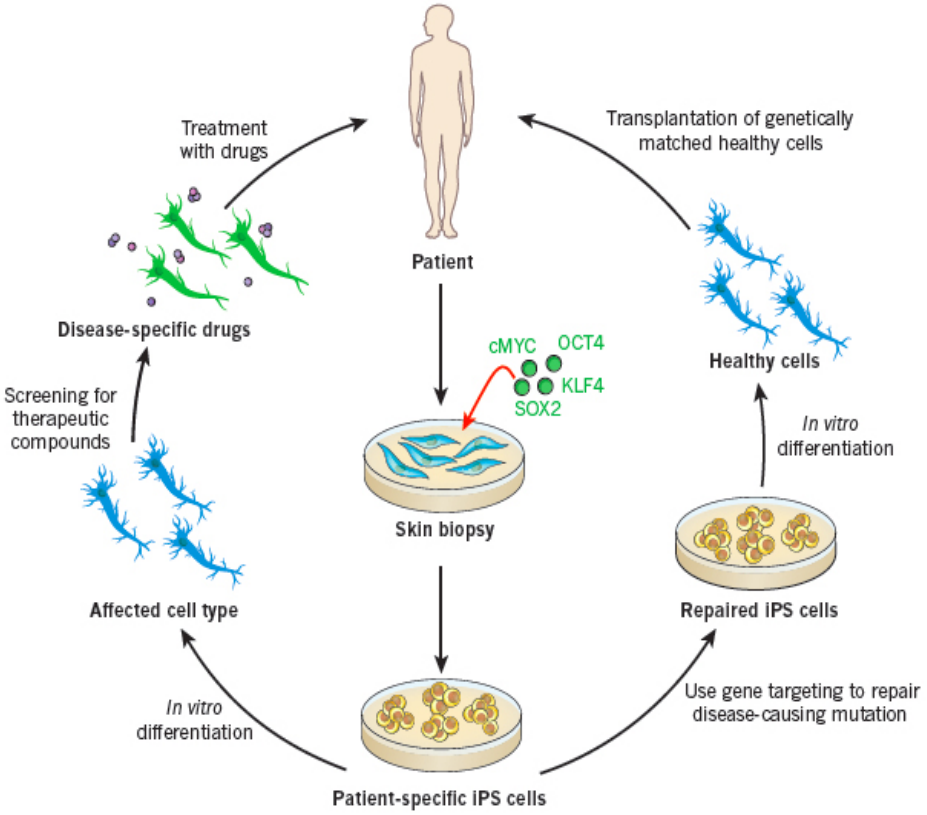


Figure 3: **Medical applications of iPS cells.** Reprogramming technology and iPS cells have the potential to be used to model and treat human disease. Modified from [141]

3. Spinal muscular atrophy

SMA is one of the most frequent genetic causes of death in childhood. It is an autosomal recessive disease that, after muscular dystrophy, is the most common neuromuscular disease. In its most severe form, disease onset occurs before 6 months of age with death of respiratory distress usually within 2 years. The major pathological characteristic of SMA is selective degeneration of lower α motor neurons in the ventral horn of the spinal cord, resulting in progressive muscle denervation, skeletal muscular atrophy, particularly of the proximal muscles, and eventual paralysis. SMA is caused by homozygous deletion or mutation of the ubiquitously expressed survival of motor neuron 1 (SMN1) gene, and the most frequent mutation is a homozygous deletion of SMN1 exon 7 [142]. In humans, there are two SMN genes, the telomeric SMN1 and its centromeric homolog SMN2, caused by the intrachromosomal duplication of 5q13. Crucially, SMN2 differs from SMN1 at base pair position 840, resulting in a C to T substitution that excludes exon 7 from approximately 85–90% of SMN2 transcripts [143]. Protein product lacking exon 7 is not functional and is rapidly degraded; therefore, the SMN2 gene produces considerably less SMN protein than the SMN1 gene. SMN is an essential cellular protein and homozygous deletion is not tolerated [144]. SMA is the consequence of variable, but low, levels of SMN produced from the SMN2 locus. The variation is due to copy number variation, and SMA disease severity correlates with SMN2 copy number [145]. SMN is an ubiquitously expressed protein of 294 AA that forms a large multiprotein complex both in the cytoplasm and in the nucleus where it is concentrated in a structure called “gems” (gemini of coiled bodies) [146]. SMN is involved in and facilitates cytoplasmic assembly of small nuclear ribonucleoprotein into the spliceosome, a large RNA-protein complex that catalyzes the splicing reaction. In the nucleus, SMN appears to be directly involved in pre-mRNA splicing, transcription, and metabolism of ribosomal RNA [147,148]. In order to test the hypothesis that AFS cells could functionally engraft in a diseased muscle, a mouse model of SMA, in which the phenotypic disease is full blown in muscle tissue, has been used [149]. In this model, the murine Smn exon 7 is flanked by two LoxP sequences (Smn^{F7}) and deletion is occurring only in skeletal myofibers by placing the Cre recombinase under the control of the human α-skeletal

actin gene promoter (HSA-Cre). Similarly to other models of muscle disease, HSA-Cre $Smn^{F7/F7}$ mice display a high proportion of myofibers with central nuclei, which lead to muscle fibers necrosis over time, heterogeneous myofiber diameters, and signs of interstitial infiltrate [149]. In contrast to the mdx mouse model, HSA-Cre $Smn^{F7/F7}$ animals manifest kyphosis, progressive muscle weakness, shrinkage, and subsequent respiratory arrest, similarly to the clinical features exhibited by the mdx/ mTR mouse model [150]. Therefore, the overall survival of HSA-Cre, $Smn^{F7/F7}$ animals is estimated to be of 10 months. After BM transplantation, the myopathic phenotype attenuates and the myofibers number and motor performance normalize up to 9 months of age [151]. However, the engraftment of BM cells in the diseased muscle tissue is poor: the cells are not able to rescue the phenotype and make a substantial, long-term therapeutic contribution [151].

4. c-kit and Stem Cell Factor

Steel factor (also known as stem cell factor, kit ligand or mast cell growth factor) is the product of the Steel locus and a member of the short-chain helical cytokine family. It has been shown by many studies to be an essential survival factor for PGC [152,153,154,155]. There are two forms of Steel protein, generated by alternative splicing: soluble Steel factor and membrane-bound Steel factor. The membrane-bound form lacks an extracellular domain containing a proteolytic cleavage site, which normally causes release of the extracellular region of the protein [156,157]. The receptor for Steel factor is the product of the W locus, c-kit, a tyrosine-kinase receptor of the PDGFRB superfamily [158,159,160]. The kit receptor tyrosine kinase (RTK) is centrally involved in the development of multiple cell lineages, including hematopoietic and germ cells, melanocytes, and the interstitial cells of Cajal (ICC) [161,162,163,164]. Insights into the roles of this receptor and its cognate ligand in these developmental processes have been greatly facilitated by the large series of naturally occurring mutations in the murine genes that encode these molecules, the dominant white spotting (W) and steel (Sl) loci, respectively. Thus, mice with loss-of-

functions in either the W or Sl loci are anemic and exhibit white spotting on the fur, sterility, and a concomitant loss of the ICC and intestinal pacemaker activity.[165]

5. Mouse amnion formation

The amnion is the innermost extraembryonic membrane that surrounds the fetus of amniotes and delineates the fluid-filled amniotic cavity, thereby providing a confined environment within the conceptus and conferring protection and shock resistance. In most amniotes, the amnion is a thin and avascular transparent membrane. In human, both amnion and chorion surround the embryo and both membranes fuse during the second trimester of pregnancy, while the yolk sac remains rudimentary [166]. In contrast, in mouse, the chorion will never fuse with the amniotic membrane after the physical separation of the amniochorionic fold shortly after gastrulation at E7.0 [167]. The chorion becomes incorporated in the chorionic disk of the placenta, whilst the amnion becomes surrounded by the visceral yolk sac, except in the part of the chorionic disk. Importantly, amnion on the one hand and yolk sac and chorionic disk on the other hand remain spaced by the fluid-filled exocoelomic cavity (Figure 4). In mouse embryos, the amnion consists throughout gestation of a simple bilayered membrane of squamous mesoderm and ectoderm, which face the exocoelomic and amniotic cavity, respectively. In the mouse embryo the epiblast gives rise to the embryo proper, as well as the amniotic ectoderm and the extraembryonic mesoderm of chorion, amnion, visceral yolk sac and allantois, respectively [168]

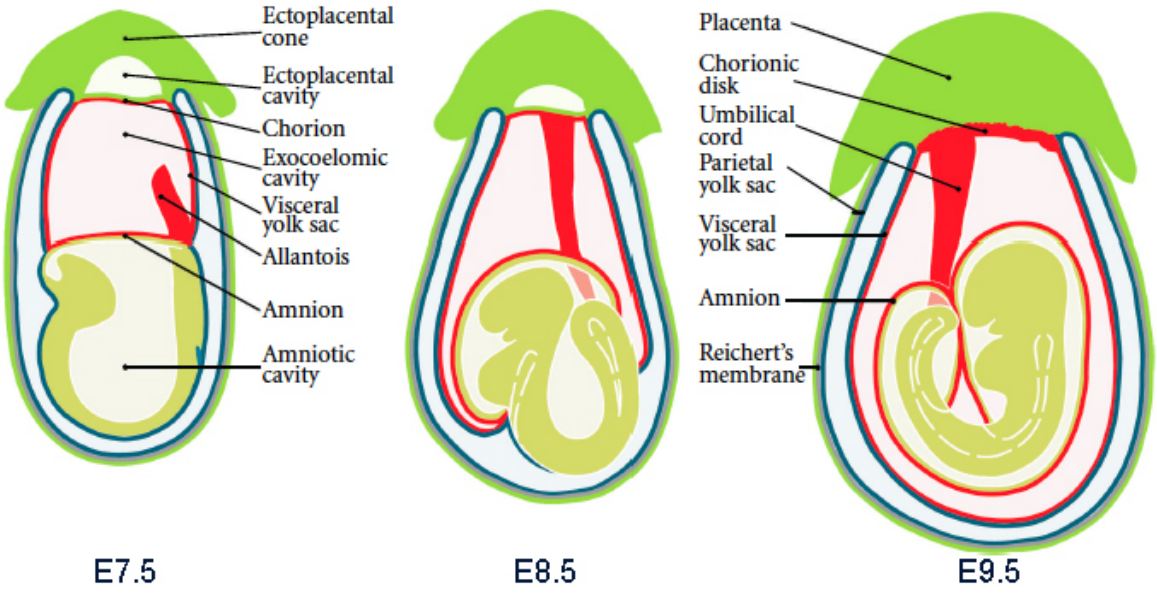


Figure 4: **Schematic representation of a mouse embryo illustrating the position of the extraembryonic tissues before and after axial rotation.** During the process of axial rotation the embryo becomes enwrapped in its extraembryonic membranes. Extraembryonic mesoderm is shown in red; yellow represents amniotic ectoderm and embryonic ectoderm (embryonic mesoderm is not depicted); green represents trophectoderm-derived extraembryonic lineages; blue shows extraembryonic endoderm. Modified from [169]

IV. Aim

Different groups have shown the potentiality of human AFS cells, but little is known regarding the mouse counterpart. The aims of this thesis are the following:

- to characterise mouse AFS cells freshly isolated;

As tool for studying the biological properties of the cells and use them in regenerative medicine field, we will perform cytofluorimetric analysis of amniotic fluid from fetuses of a wide window of gestational ages.

- to evaluate the myogenic potential of mouse AFS after transplantation in a mouse model of SMA, HSA-Cre, $Smn^{F7/F7}$;

To explore the therapeutic efficiency of murine AFS cells. A well established model of muscle disease will be used as pre-clinical model: a muscle mutant of SMA (HSA-Cre,$Smn^{F7/F7}$) where skeletal muscle is the direct damaged tissue.

- to investigate their PGC origin;

In the past, different groups identified in genetically modified mice three systems influenced by the absence of c-kit: 1) PGC, 2) neural crest cells and 3) HSC [165]. In particular the attention will be done on the hypothesis that mouse AFS could be PGC that during the migration go through the amniotic fluid. This hypothesis has been based on data obtained with human AFS; it has been demonstrated that human AFS cells, obtained from AF of the first-trimester of gestation, express PGC markers (FRAGILIS, SSEA1, TNAP, NANOS, BLIMP1, PUM2, STELLA, DAZL, and VASA), and have the ability to reprogram to pluripotency by culture on Matrigel in human ES Medium supplemented with the histone deacetylase inhibitor (HDACi) valproic acid (VPA) [71].

- to induce the pluripotency using a non-viral method (PiggyBac, PB).

The technique of obtaining iPS really represent a new avenue for using pluripotent stem cells and a non-viral method will be tested to reprogram mouse AFS cells, using the PB system.

V. Materials and Methods

1. Mice

Male mice and female mice of the diffent models (C57BL6/J, HSA-Cre $Smn^{F7/F7}$, C57BL6/GFP, TNAP/Cre, Oct4-GFP, Z/Red, Z/eGFP and Tomato) used were mated to obtain the fetuses from which the amniotic fluid has been obtained. All procedures were approved by the University of Padua/Toronto's Animal Care and Use Committee and, in accordance with Italian and Canadian law, were communicated to the Ministry of Health and local authorities.

2. Amniotic fluid collection and stem cell selection

Embryo age was defined relative to the morning of vaginal plug discovery (E0.5). All dissections were performed under a stereomicroscope (Leica Microsystems). Amniotic fluid samples were harvested from pregnant mice between embryonic days from E10.5 to E16.5. Amniocentesis has been done following an accurate procedure: first there is the removal of the maternal uterine wall, to expose the yolk sac. At this point the chorion and yolk sac are carefully removed. Amnion rupture result in amniotic fluid leakage, and amniotic fluid is harvested with a syringe fitted with a 28-gauge needle. Murine amniotic fluid-derived c-kit^{+} cells were isolated using the Miltenyi Mouse Lineage Cell depletion kit and the CD117 MicroBeads kit (all from Miltenyi Biotech).

3. Flow cytometry analisys

Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences) with CellQuest acquisition software (BD Biosciences) or a Gallios flow cytometer (Beckman Coulter). The antibodies used were CD177 APC (Biolegend) or CD117 PE Cy5 (eBioscience), Sca1 FITC, CD90 FITC, CD44 FITC, CD34 PE and CD105 PE (All from BD Biosciences), CD31 FITC, Flk1 PE, CD45 PE (all from BD Biosciences) and the 7AAD (BD pharmingen) or DAPI as viability probe.

4. *In vitro* hematopoietic differentiation protocol

Myeloid and erythroid potentials were assessed by cell culture in a semisolid medium. Around 500 cells were gently mixed with 1 mL MethoCult M3434 methylcellulose colony assay medium (StemCell Technologies) and cultured for 11 days in 24-well plates at 37°C in humidified 5% CO_2 air. Colonies consisting of at least 50 cells were counted every week and classified according to morphology and color of the colony and the single cells, using an inverted Olumpus IX71 microscope (10x magnification). Pictures of colonies were taken with a Olympus Camera C3040 and processed with analySIS software.

5. Real Time PCR

In order to quantify the different amount of Oct4, Sox2, c-Myc, Klf4, Nanog, Sca1, c-kit and Pax7 mRNAs in freshly isolated cells or muscle samples, total RNA has been extracted using RNeasy Plus Mini kit (QIAGEN GmbH) following the supplier's instructions. RNA has been then quantified with a ND-1000 spectrophotometer and 1ug has been retrotranscripted with SuperScript II and related products (all from Invitrogen) in a 20 ul reaction. Real-time PCR (qRT-PCR) reactions were performed using a LightCycler II (Roche). Reactions have been carried out in triplicate using 4 ul of FASTSTART SYBR GREEN MASTER (Roche) and 2 ul of primers mix FW plus REV (final concentration, 300/300 nM) in a final volume of 20 ul. Serial dilutions of a positive control sample have been used to create a standard curve for the relative quantification. The amount of each mRNA has been normalized for the content in β2-microglobulin. Primer sequences were the following: c-kit FW: TGGTCCGCTGCCCTCTGACA, c-kit REV: CCTTGATGGCTGCCCGCACT, Pax7 FW: AGCAAGCCCAGACAGGTGGCG, Pax7 REV: GGCACCGTGCTTCGGTCGCA, Oct4 FW: TGGAGGAAGCCGACAACAATGAGA, Oct4 REV: TGGCGATGTGAGTGATCTGCTGTA, Nanog FW: CCCTTCCCTCGCCATCACACTG, Nanog REV: GGAAGGGCGAGGAGAGGCAGC, Sox2 FW: TCGGGGAAGCGTGTACTTAT, Sox2 RW: CATGCACAACTCGGAGATCA, β2-microglobulin FW: GCTTCAGTCGTCAGCATGG, β2-microglobulin RW: CAGTTCAGTATGTTCGGCTTCC, Klf4 FW:

TGCCCCGACTAACCGTTGGCGT, Klf4 REV: GCTGCACCAGCTCCGCCACT, c-Myc FW: TGCCCGCGATCAGCTCTCCT, c-Myc REV: CGTGGCTGTCTGCGGGGTTT

6. Single Cell Analisys

(a) Single cell deposition

Mouse AFS cells have been sorted for ckit using a FACS Aria I Sorter equipped with an automatic cell deposition unit (BD Bioscience). 7-Aminoactinomycin D (BD Bioscience) was added as a viability marker in the sorting procedure. Cells have been collected in 96-well plates for molecular biology containing 5 µL of PBS-DEPC 0.1%, and stored at -80°C.

(b) Single cell PCR.

Mouse AFS cells isolated as single cells were analysed using protocols described in [170]. Primers sequences were the following: c-kit FW_A: GATCCCGACTTTGTCAGATG, ckit REV_B: TTTGGGACAAACGTCAGGTC, c-kit FW_C: ACACGTGCAGCAACAGCAAT, c-Myc FW_A: AAACTTTGCCCATTGCAGCG, c-Myc REV_B: CCTCGTCGCAGATGAAATAG, c-Myc FW_C: TCCGGGGAGGGAATTTTTGT, Klf4 FW_A: CACCCACACTTGTGACTATG, Klf4 REV_B: TACTGAACTCTCTCTCCTGG, Klf4 FW_C: AAATTCGCCCGCTCCGATG, Nanog FW_A: CCACAGTTTGCCTAGTTCTG, Nanog REV_B: GACCTTGTTCTCCTCCTCCT, Nanog FW_C: CTTACAAGGGTCTGCTACTG, Oct4 FW_A: CTCTTTGGAAAGGTGTTCAG, Oct4 REV_B: CTCGAACCACATCCTTCTCT, Oct4 FW_C: TGGAGGAAGCCGACAACAAT, sca1 FW_A: AGAGGGCTCCAGGAAGAATT, Sca1 REV_B: TCTGTGTTACTCAGGAGGCA, SCa1 FW_C: ATCCTGGGTACTAAGGTCAA, sox2 FW_A: CATGGGCTCTGTGGTCAAGT, sox2 REV_B: CCTACTCTCCTCTTTTTGCAC, sox2 FW_C: GGGACATGATCAGCATGTAC, 28S FW_A: ACAGTGATGGTTCAGGAGGG, 28S REV_B: AAGTAACAGAACTTGGCTGG, 28S FW_B: CGCTACTATGAGAAGCCTTG.

7. Teratoma assay

For teratoma assay 10^5 mouse AFS cells were injected into the muscle of the hindlimb of $Rag2^{-/-}\gamma c^{-/-}$ mice. Transverse sections (8–10 um thick) of isopentane-frozen muscles injected were stained with hematoxylin and eosin to evaluate the tumors presence.

8. AFS cell expansion and PGC reprogramming

For the expansion freshly isolated AFS cells were plated onto a feeder layer of mitomycin C-treated mouse embryonic fibroblasts SNL (applied Stem Cells Inc.) in DMEM knockout (Invitrogen) supplemented with 15% heat-inactivated FBS (Invitrogen), 0.1 mM nonessential aminoacids (Invitrogen), 2 mM L-glutamine (Invitrogen), 50 U (ug)/ml penicillin/streptomycin (Invitrogen), 0.01 mM 2-mercaptoethanol (Sigma), 10 ng/ml BMP4 (R&D Systems), and 20 ng/ml LIF (Sigma). Cells were cultivated at 37°C and 5% CO_2. For PGC reprogramming freshly isolated AFS cells, or genital ridges obtained from embryos Oct4GFP positive at E10.5-12.5, were trypsinised to a single cell suspension and plated onto a feeder layer of mitomycin C-treated Sl^4-m220 (from Dr. Matsui from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Japan.) in DMEM High Glucose (Invitrogen) supplemented with 15% heat-inactivated FBS (Invitrogen), 2mM Glutamax (Invitrogen), 50 (ug)/ml penicillin/streptomycin (Invitrogen), 0.1mM sodyum pyruvate (Invitrogen), 0.01mM 2-mercaptoethanol (Sigma), 25 ng/ml FGF-2 (R&D Systems) and 1000U/ml LIF (Millipore) for the first two days and for the following days in 2iLIF medium composed of 50% DMEM F-12 (Invitrogen), 50% Neurobasal Medium (Invitrogen), 1% Knockout Serum Replacement (Invitrogen), 2mM Glutamax (Invitrogen), 50 (ug)/ml penicillin/streptomycin (Invitrogen), 0.01mM 2-mercaptoethanol (Sigma), N2 (1x) and B27 (1x)1x supplement (Invitrogen), BSA Fraction V 7.5% (Invitrogen), 1000U/ml LIF (Millipore), MEK inhibitor PD0325901 (1 uM) and GSK3 inhibitor CHIR99021 (3 uM) (both from Selleck Biochemical).

9. Cell Injection

Three-month-old HSA-Cre, $Smn^{F7/F7}$ mice were randomized to receive either no treatment, mouse BM cells, or mouse AFS cells. Approximately 25,000 $GFP^{+/+}$ cells were injected via tail vein in the AFS cells group and 50,000 $GFP^{+/+}$ cells were injected via tail vein in the BM group. *In vitro* expanded AFS cells were sorted with an Aria FACS system (Becton Dickinson) and selected for the expression of GFP and c-kit prior to injection. After sacrifice (1 month post-transplantation (pt)), muscle differentiation and regeneration were investigated by assessing the percentage of cells derived from donor $GFP^{+/+}$ cells in recipient muscles, considering the total number of muscle fibers (both centrally nucleated and with peripheral nuclei).

10. Muscle physiology, histology, and immunofluorescence analyses

In vivo determination of gastrocnemius strength and contraction kinetics was carried out as described previously [171]. Transverse sections (8–10 um thick) of isopentane-frozen skeletal muscle (Tibialis Anteriour, TA) of 3-month- old transplanted mice were stained with hematoxylin and eosin and Masson's trichrome. To evaluate the total number of centrally nucleated muscle fibers, serial 40-um sections of the entire TA muscle were prepared. Immunostaining of GFP or dystrophin was performed using rabbit anti-GFP (1:200; Invitrogen) or anti- dystrophin (1:150; Abcam). Sections were mounted with Vecta-shield and 4,6-diamidino-2-phenylindole (Vector Laboratories), observed under an Olympus BX60 microscope (Olympus), and pictures taken using Viewfinder Lite software.

11. *In Vivo* imaging

Prior to imaging, mice were anesthetized (with a mixture of Rompum and Zoletil given i.p.), shaved and depilated to completely remove hair, and imaged on the eXplore

Optix time-domain imager (ART, Montreal, Quebec). Image processing and data analysis were performed using explore Optiview 1.04 software (ART).

12. DNA extraction and PCR analyses

DNA from organ and tissues samples was extracted with a DNeasy Blood & Tissue kit (QIAGEN GmbH) and then quantified with a ND-1000 spectrophotometer (Thermo Scientific). Genomic DNA samples extracted from GFP^+ AFS cells and from WT organs were, respectively, used as positive and negative controls for the amplification of the GFP gene. PCR reactions for the TERT and GFP genes were carried out as previously described [172]. Primers sequences were the following: GFP FW: TGAACCGCATCGAGCTGAAGGG, GFP REV:TCCAGCAGGACCATGTGATCGC, TERT FW: ACCCACTATCCTTGTGGTGCATGA, TERT REV:AGATCGAGCAGCTGCAAGACCATA.

13. PB production

PB expression vectors were generated using standard cloning procedures. The plasmid DNA for mouse AFS transfection was prepared using a QIAprep Spin Miniprep Kit and Maxiprep Kit (Qiagen).

14. Transfection, iPS cell generation and culture

Mouse AFS cells were expanded like described in point 7 (AFS cell expansion and PGC reprogramming) and transfected after 7 days of culture, with the Yamanaka's factors (Oct4, Klf4, cMyc, Sox2). Factors were in an ORFs linked with 2A peptide sequences, into the PB-TET transposon plasmid (called PB-TET-OKMS) under the transcriptional control of the tetO2 tetracycline/doxycycline inducible promoter. All was linked to the gene of a cherry fluorescent protein through an IRES sequence. Cells were transfected with the PB-TET-OKMS transposon plasmid, in conjunction with a PB transposase expression plasmid (mPBase) and with the reverse tetracycline transactivator (rtTA) transposon plasmid. Fugene HD Transfection Reagent (Promega) has been used, with a ratio DNA :Transfection Agent = 1ug : 4ul. The ex-

pression of the Yamanaka's factors were induced with 1.5 ug/ml doxycycline (Sigma) the day after the transfection. Twenty-four hours after transfection, cells were fed with fresh ES media daily without passage. The resulting colonies were picked 30 days after transfection and iPS clones were maintained on inactivated feeders in ES medium.

15. Alkaline phosphatase staining and immunfluorescence

Alkaline phosphatase staining was carried out following the manufacturer's instruction (Vector Red Alkaline Phosphatase Substate Kit I). Immunofluorescence analyses were performed using as primary antibodies SSEA1 (1:100, Millipore) and Nanog (1:100, ReproCells) . Secondary antibodies were respectively Alexa564-conjugated goat anti-mouse IgM (1:200) and Alexa594-conjugated chicken anti-rabbit IgG (1:200) (all from Life Technologies). Primary and secondary antibody immunofluorescence were performed according to standard protocols. Nuclei were stained with DAPI solution in PBS 1X (Life Technologies).

16. Statistical analysis

Values were reported as means ± SD or SE. Statistical significance of the differences between means were assessed by analysis of variance followed by the Student−Newman−Keuls test or by Student's t−test for paired or nonpaired data. Statistical significance wiere set at $P < 0.05$.

VI. Results

1. Mouse AFS cells characterisation

Mouse amniotic fluid was collected from offspring of female $GFP^{+/+}$ and males $GFP^{-/-}$, and all the analysis were performed on cells selected for the GFP expression to avoid any maternal contamination. On the amniotic fluid collected a lineage depletion for the hematopoitic lineage was done, to remove all the mature cells, and after that a selection for the markers c-kit. The selection for this marker was done beacuse c-kit have been previously identified as marker of stem cells in the human AF. [62]. Within the lineage-negative fraction, some c-kit positive cells were detected in a variable proportion according to the gestational age (Figure 5).

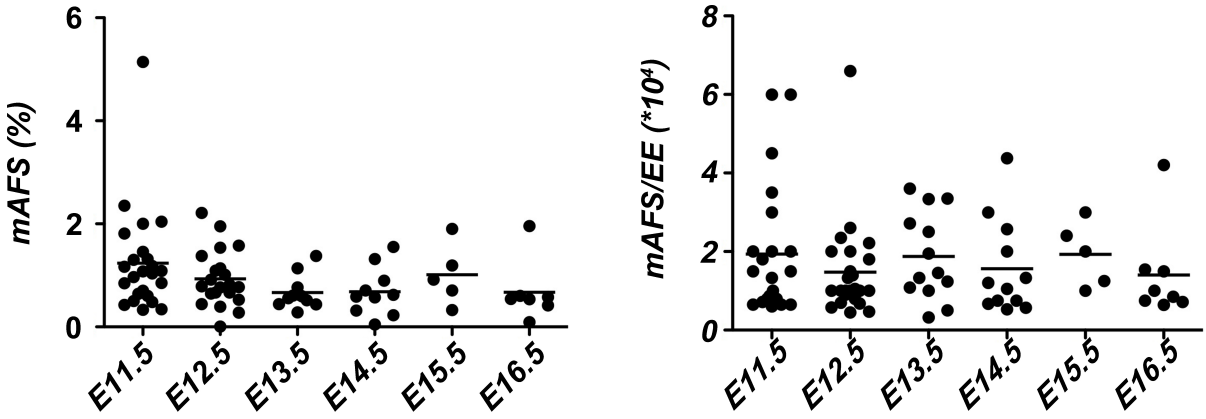


Figure 5: **Presence of mouse AFS cells in the amniotic fluid.** The percentage of mouse AFS cells as a function of the gestation stage is indicated in the left panel. The right panel shows the total number of mouse AFS cells per embryo equivalent (EE). Means are represented by bars.

Along the period of gestation there is a peak in number of mouse AFS cells present in the amniotic fluid at E11.5 and then they slowly drecrease. The total number of mouse AFS cells in the mouse AF per embryo equivalent is around 10.000-20.000 cells for embryo (Figure 5, right panel).

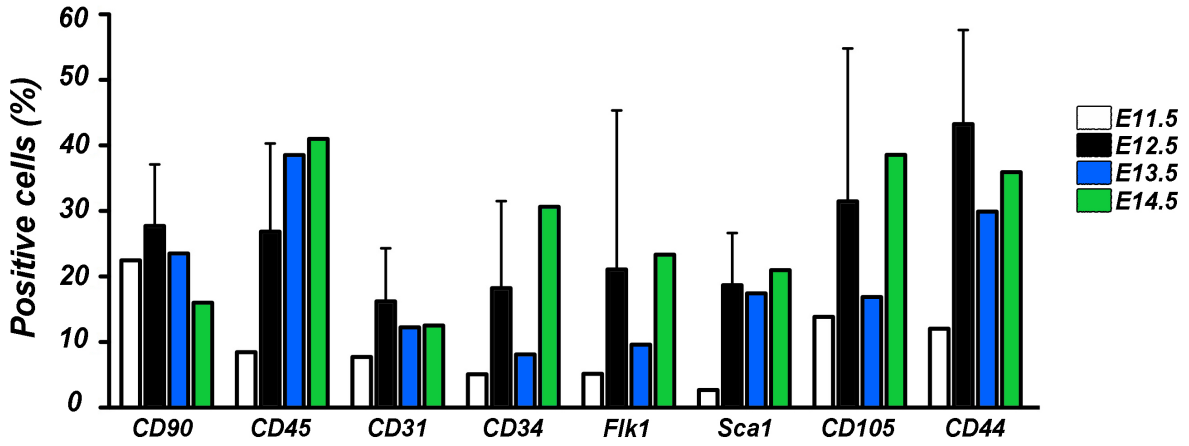


Figure 6: **Phenotypic characterisation of mouse AFS cells.** Mouse AFS cells were stained with antibodies specific for CD90, CD45, CD31, CD34, Flk1, Sca1, CD105 and CD44. The histograms show the expression of the markers at different embryonic stages (from E11.5 to E14.5, for E12.5 experiments n=3).

An immunological phenotype was performed to characterize the mouse AFS that express markers characteristic of hematopoietic (CD45, CD34, Sca1), mesenchymal (CD90, CD105) cells together with CD44,CD31 and Flk1, and their expression change during the course of gestation (Figure 6). Considering the ckit$^+$ population of the AF CD90, a marker of the mesenchymal stem cells, was expressed from 20-25% of ckit$^+$ cells at E11.5-13.5 and its expression decreased to 16% at E14.5; CD45, the marker that define the commitment to the hematopoietic lineage, was expressed by 8.5% of cells and then it increased during the gestation, reaching 40% of expression at E14.5. CD31 (Platelet Endothelial Cell Adhesion Molecule 1, Pecam-1) is a marker expressed by endothelial cells, and its expression in the ckit$^+$ cells ranged from 7% to 12%. CD34 is an hematopoietic cells marker, differently expressed during the gestation since there was an expression from 5% to 8% at E11.5 and E13.5, respectively, and an higher expression at E12.5 and E14.5 (18% and 30%). In the same way Flk1, the receptor for vascular endothelial growth factor (VEGFR), changed during the gestation, indeed it was expressed from 5% to 9% of cells at E11.5 and E13.5, while increased at E12.5 and E14.5 (21% and 23%). Sca1 is a characteristic marker of HSC and different adult stem cells, it was expressed by a low number of cells at E11.5 (around 2% of cells) and reached 20% at E14.5. CD105 is another marker of mesenchymal stem cells, it was expressed by 14%, 31%, 17% and 38% of the c-kit$^+$ cells at the different embryonic stages. CD44 is a cell surface glycoprotein involved in cell-

cell interactions, in the AF was expressed by 12% of c-kit^{+} cells at E11.5, and then its expression increased during the course of gestation. Two distinct populations have been discoverd in the AF, they differ for the intensity of expression of ckit, as it is shown in figure 7, and they have been called c-kit^{high} and c-kit^{low}. They can be easily distinguished for the expression of the marker c-kit, a magnitudo of intensity that ranged from 10^1 to 10^2 identify the ckit^{low}, while the c-kit^{high} had an intensity of expression from 10^2 to 10^4 (Figure 7A). They differed also for their presence in the AF during the course of gestation (Figure 7B); it has been evaluated that the c-kit^{low} were predominant (99%) at E11.5, while at this stage the number of c-kit^{high} was very low (1%). In particular during the course of gestation the number of the c-kit^{low} decreased and the one of c-kit^{high} increased, in fact at E16.5 c-kit^{low} were 60% and c-kit^{high} were 40%. These two subpopulations have been studied under the immunological phenotype (Figure 7C), and it has been shown that they differ for the expression of several markers. Notably the two populations had an opposity phenotype regarding the expression of markers, like CD44, CD45 and CD90. c-kit^{high} were negative for Flk1, Sca1 (except for < 1% of cells positive at E14.5) and CD105 (except for 5% of cells positive at E14.5), and there were few cells positive for CD90, CD31 and CD34. c-kit^{low} were positive for all the markers evaluated in all the considered embryonic stages, with different trends during the different days. To investigate if a different phenotype correspond to a different functional potentiality the hematopoietic differentiation has been studied at the embryonic stage E12.5 for the two populations. Mouse AFS cells c-kit^{high} and c-kit^{low} were sorted and cultured in semisolid medium to assess their hematopoietic function (Figure 8). The two populations exhibited the same clonogenic potential (18 colonies per 500 cells, ie, 3.6%).

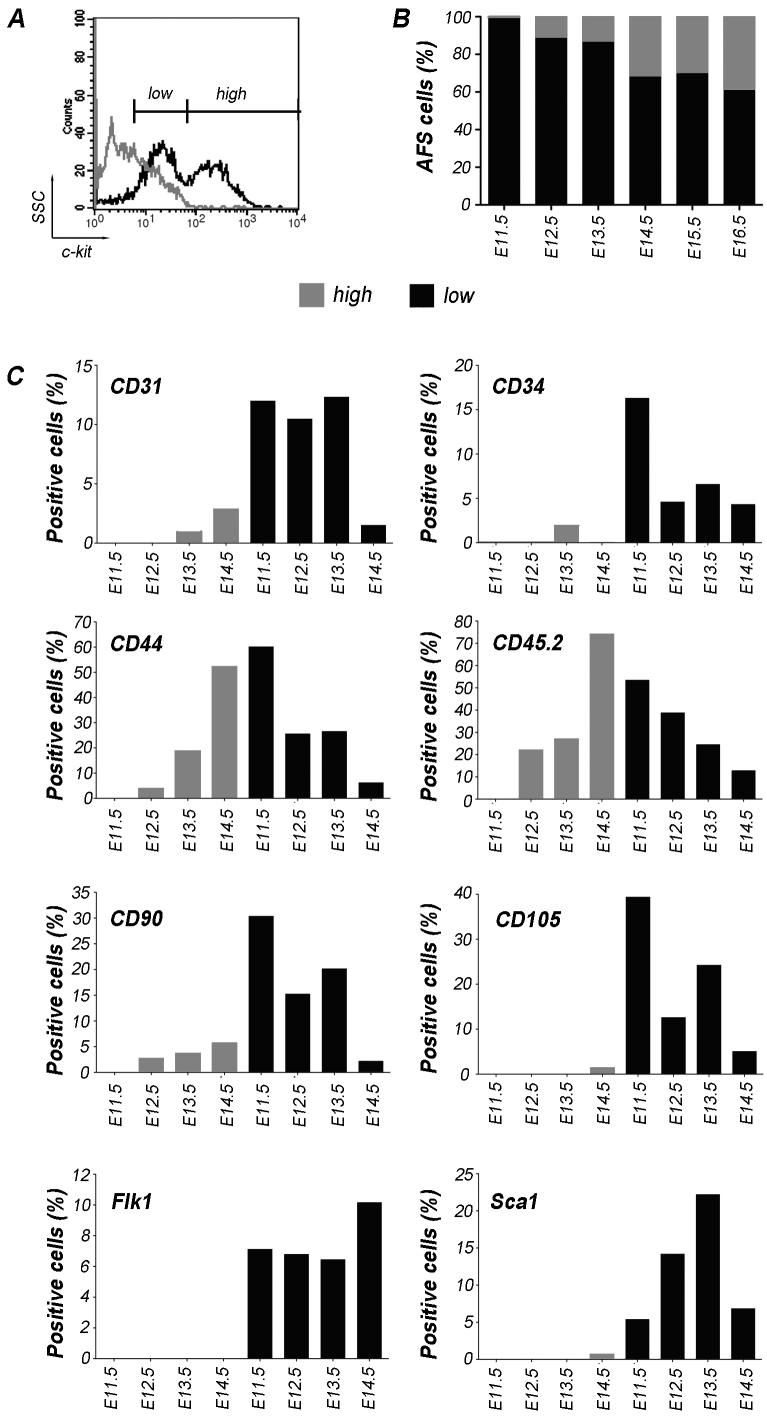
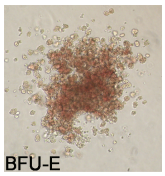
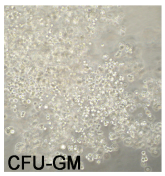


Figure 7: **Characterisation of c-kit^{high} and c-kit^{low} population in the mouse amniotic fluid.** (A) mouse AFS cells were analyzed by flow cytometry and two population were distinguished on the basis of intensity of expression of the marker c-kit. (B) c-kit^{high} and c-kit^{low} change in number during the course of gestation. (C) Immunological phenotype on the basis of expression of different markers (CD31, CD34, CD44, CD45, CD90, CD105, Flk1 and Sca1).

	c-kit^{high}	c-kit^{low}
BFU/CFU-E	28%	19%
CFU-GM	55%	68%
CFU-GEMM	17%	13%





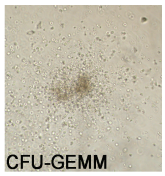


Figure 8: ***In vitro* hematopoietic differentiation.** The percentages of the different types of colonies generated, obtained 11 days after seeding 500 candidate cells is shown. Representative images of day 11 mAFS cell–derived hematopoietic BFU-E, CFU-M, and CFU-GEMM cells (cultured in semisolid methylcellulose-based medium supplied with hematopoietic cytokines) are shown.

These colonies included erythroid colony- and burst-forming units (CFU- or BFU-E) and granulocyte/macrophage CFUs (CFU-G/M/GM). The proportion of mixed colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) cells was similar in the two analyzed population , thus attesting to the presence of multipotent hematopoietic progenitors (Figure 8). As mentioned before human AFS were characterized for their expression of pluripotency markers like Oct4 and SSEA4, in addition to their capability to differentiate into cells of the three germ lineages [62]. Mouse AFS cells were also studied for the expression of pluripotency markers Oct4, Sox2, Nanog, c-Myc and Klf-4. Real Time-PCR analysis showed that the expression of these markers was very low, excepted for the genes c-Myc and Klf-4 at E11.5 (Figure 9).

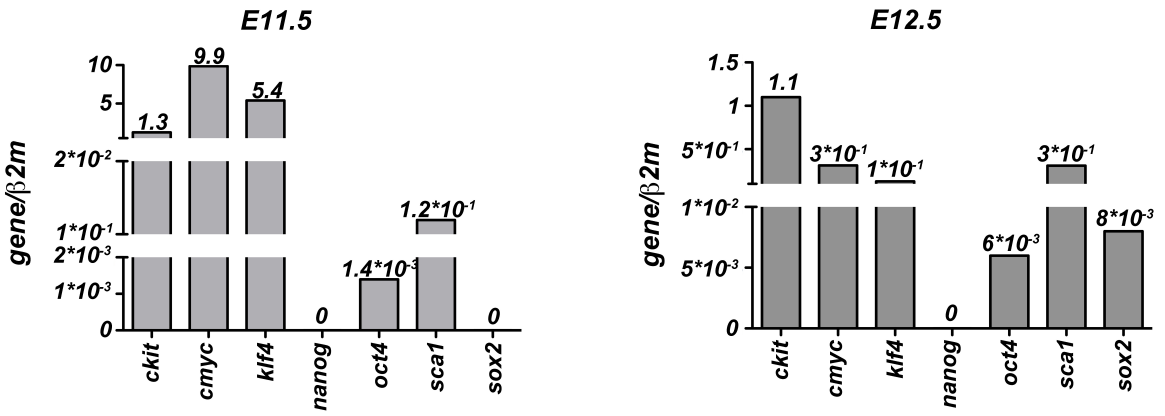


Figure 9: **Gene expression analisys of pluripotency markers**. RT-PCR analyses were performed to investigate the expression of pluripotency genes at two different embryonic stages (E11.5 and E12.5). Values are expressed in arbitrary units as gene/β2microglobulin ratio, and considering 1 the level of expression of mouse ES cells.

Quantitative gene expression analysis determine the average gene expression within a population, overlooking possible cell-to-cell heterogeneity that could lead to different cell behaviors or fates. Understanding individual cell behavior requires multiple gene expression analyses of single cells, and may be fundamental for the understanding of all types of biological events and/or differentiation processes. To deeper investigate the gene expression of the pluripotency markers, single-cell PCR analysis has been performed at different embryonic stages. All the attained data are summarized in the Figure 10. The single cells analysis confirmed that mouse AFS cells did not express Nanog, as showed with the Real Time-PCR analisys at population level. Regarding the other pluripotency markers c-Myc, Klf4 and Sox2 were expressed in all the analyzed embryonic stages however the expression levels were not always the same; at E10.5 the number of positive cells for c-Myc, Klf4 and Sox2 were 70%, 30% and 11% respectively, at E11.5 were 84%, 29% and 4%, at E12.5 were 16%, 87% and 13%, at E13.5 were 45%, 55% and 10%, at E14.5 were 85%, 4% and 2%. It is worth of notice that Oct4 gene was not expressed at E10.5 and E14.5, was low at E11.5 and E12.5 respectively 2% and 11%, while was expressed in the 76% of cells at E13.5. Summarizing, the embryonic stage of E13.5 seems to be the best temporal window to find mouse AFS cells expressing the pluripotency genes Oct4, Sox2, c-Myc and Klf4 on a considerable numbers of cells (76%, 38%, 55% and 45%).

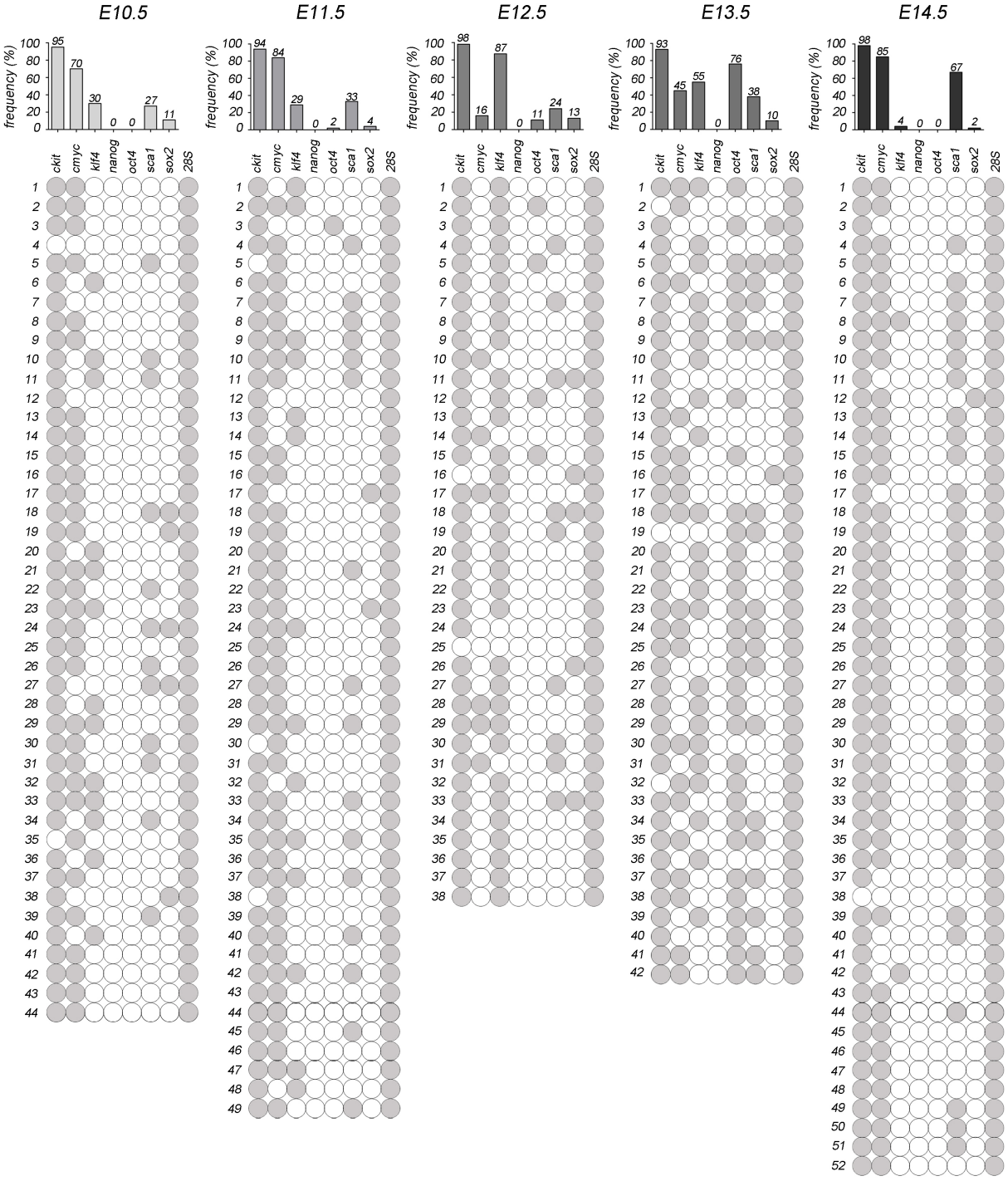


Figure 10: **Frequency of expression of plurypotent genes.** Single-cell multiplex RT-PCR analyses were performed to investigate the frequency of expression of pluripotent genes at different embryonic stages (E10.5-E14.5). In all cases 28S was used as an endogenous control. The results are shown as histograms representing the proportion of cells expressing each gene. Single mAFS cells were sorted and analyzed by multiplex RT-PCR, as described in “Methods.” Each row shows the same (numbered) individual cell. Each column shows a different gene. Empty symbols represent cells not expressing that particular mRNA; gray symbols, positive cells where mRNA levels were not quantified.

Also Sca-1 was expressed by a variable percentage of cells (27%, 33%, 24%, 38% and 67%) at the different embryonic stages analyzed. Cells that co-expressed genes at the different embryonic stages are summarized in the table below:

	E10.5	E11.5	E12.5	E13.5	E14.5
ko	0%	0%	10%	43%	0%
ks	0%	0%	13%	5%	0%
km	11%	18%	8%	2%	2%
kom	0%	0%	0%	0%	0%
koms	0%	0%	0%	0%	0%
sm	7%	2%	0%	2%	2%
so	0%	0%	0%	5%	0%
om	0%	0%	0%	33%	0%
kos	0%	0%	0%	5%	0%

Table 2: **Frequency of coexpression of pluripotency genes in mouse AFS cells.**

A test considered the gold standard for the evaluation of the pluripotency is the teratoma *in vivo* fomation. These tumor masses that contain differentiated derivatives of all three embryonic germ layers, grow when pluripotent cells are injected in immunocompromised mice. [173]. Pluripotency of mouse AFS cells has been evaluated after injection of about 10^5 cells into the muscle of the hindlimb of $Rag2^{-/-}\gamma c^{-/-}$ mice. After six weeks from the injection, there were no presence of teratoma (data not shown). To summarize, (1) mouse AFS cells are an heterogenous population and their characteristics change during the course of gestation but they do not form teratoma, (2) they expressed the majority of the pluripotency markers (all the Yamanaka's factor plus Sca-1) at the embryonic stages 13.5, as evalueted by the single cells gene expression analysis, (3) they also express hematopoietic and mesenchymal stem cells markers as shown by the flow cytometry analysis, finally (4) the cells possess hematopoietic potential, as demostrated by the methylcellulose cell culture of the two populations c-kit^{high} and c-kit^{low}, confirming previous data [64].

2. Myogenic potential of mouse AFS cells

To evaluate the myogenic potential of the mouse AFS cells a mouse model of SMA called HSA-Cre, $Smn^{F7/F7}$ has been used. As previously described in this model the defect is localized only in the muscle, no other tissues are affected from the genetic ablation. Three months old mice were choosen for the transplantation, because at this time the muscle defects is evident. Mice were divided into three groups: ones that received the tail vein trasnplantation with mouse AFS cells (25.000 cells), ones with mouse BM cells (50.000 cells), both obtained from GFP^+ donator and one without transplantation (untreated). The survival of the treated mice was evaluated, mice not treated die at the age of 10 months, while at that time mice treated with mouse AFS or BM cells had a survival rates of 75% and 50% respectively (Figure 11 a). The treated mice differed from the untreated animals on respect to their force, in fact *gastrocnemius* contraction kinetics was measured blinded 1-month after the transplantation and this analysis showed that both BM- and AFS-treated mice had levels of strength comparable to the ones obtained with wild type (WT) mice (Figure 11b). Consistently with survival and muscle strength, AFS-treated mice were similar to wild type mice in terms of weight, movement, and skeletal anatomy as confirmed at MicroCT scan analysis (Figure 11c-e). On the contrary, HSA- Cre, $Smn^{F7/F7}$, which did not receive any treatment, showed pronounced kyphosis with impaired movements by the age of 8 months (Figure 11d).

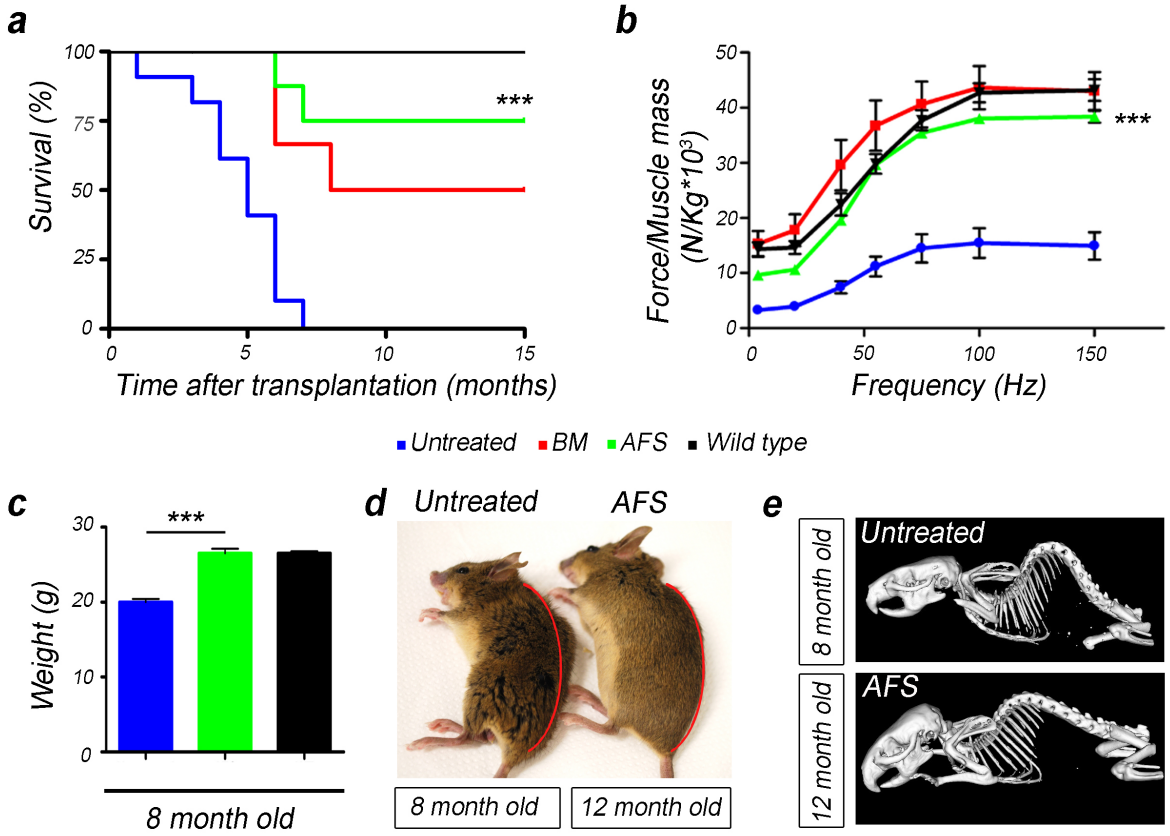


Figure 11: **Transplanted animals improve in survival and clinical parameter**. (a) Survival curve: AFS-treated mice increased life expectancy both when compared with BM-treated and -untreated animals. Untreated=10 animals, BM=8 animals, AFS=8 animals (***,p < .001). (b) Muscle strength analyzed at different frequencies. Normalized force frequency on muscle mass displays that both BM- (n=8) and AFS-treated (n=8) mice had 75% more muscle strength than untreated (n=6) animals 1 month after transplantation (***, p < .001). (c): Body-weight of untreated (n=4), AFS-treated (n=4), and WT (n=8) mice at 8 months (***, p < .001). (d): The kyphosis, which is present in the untreated mice from 3 months of age, disappeared in AFS-treated mice. (e): MicroCT scan of skeletal anatomy of untreated and AFS-treated mice confirmed the different curvature of the spine. Abbreviations: AFS, amniotic fluid stem; BM, bone marrow; WT, wild type.

Following morphological muscle analyses performed 1-month pt (i.e., 4 months of age; Figure 12a), in both BM- and AFS- treated mice, the histological aspect of the muscle tissue resulted indistinguishable from WT, with few central nucleated fibers (<1%, comparable to WT mice; Figure 12 a, e). In contrast, untreated animals presented a very large number of central nucleated fibers (mean value: 63.70% ± 0.41%, p < .001; Figure 12 a, e). Masson's trichrome staining showed that untreated animals presented large connective tissue areas between fibers, which were absent in both WT and cell-treated mice. Immunofluorescence analyses clearly indicated

that the morphological appearance of muscle in BM- and AFS-treated animals was correlated with a relevant proportion of GFP^+ fibers (31.57% ± 7.04% and 37.86% ± 9.48%, respectively for BM and AFS). Changes in the muscle tissue were also confirmed by the observed distribution of dystrophin expression, which was similar only in transplanted and WT mice but differed in the untreated animals (Figure 12a). Despite the similarity between BM- and AFS-treated animals in terms of physiology and number of central nucleated and GFP+ myofibers, the regenerative index, defined as the number of donor-engrafted myofibers generated per 10^5 transplanted cells, differed significantly between BM- (1,263.00 ± 282.76) and AFS-treated mice (3,029.16 ± 758.71; $p < .001$), respectively (Figure 12b). Additionally, RT-PCR revealed that 1 month after treatment, in the TA muscles of AFS-treated mice, there was higher expression of Pax7 than in untreated or BM-treated mice ($p < .05$; Figure 12c). Further evidence of the differences between the two groups was confirmed by analysis of surviving animals at 15 months post-transplantation (Figure 12d). Morphological analysis of samples from BM-treated mice displayed a high number of central nucleated fibers (39.90% ± 17.68%) and consistent infiltration of interstitial tissue between the myofibers, a situation that differed greatly from the one observed in AFS-treated mice. Moreover, at this stage (15 months pt), no GFP^+ fibers were found in BM-treated animals, whereas 58.00% ± 2.43% of myofibers were GFP^+ in AFS-treated mice (Figure 12e). This finding highlights the sustained effect of AFS cells transplantation.

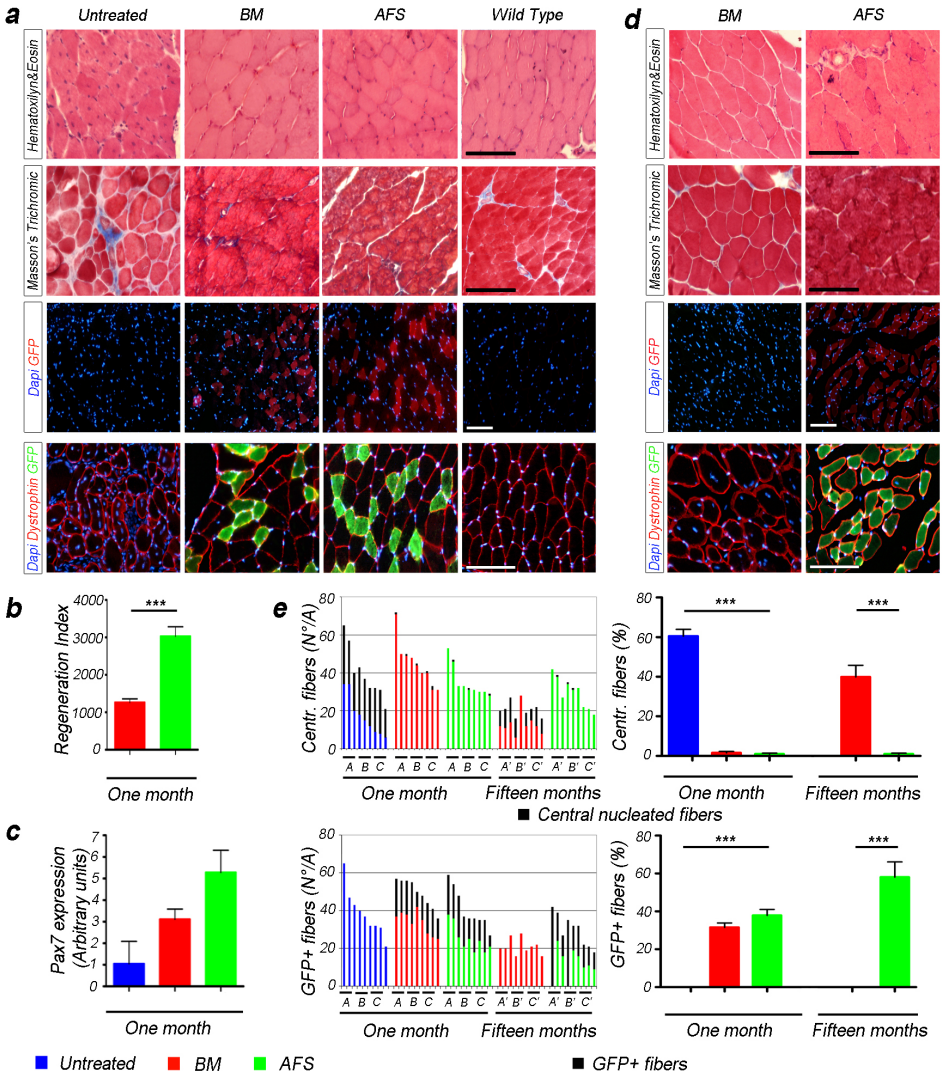


Figure 12: **Restoration of muscle phenotype in mutant mice.** (a): Three-month-old HSA-Cre, $Smn^{F7/F7}$ mice were transplanted alternatively with BM or AFS cells and sacrificed 1 month after treatment for analyses. One month after transplantation, hematoxylin and eosin staining reveals a normal appearance of the TA muscle structure after receiving either BM or AFS cells. On the contrary, TA muscle of untreated animals present fibers disarrangement (dystrophin staining) with interstitial tissue deposition identified at Masson's trichrome. Scale bar=100 um. (b): Regeneration index indicates that AFS cells are more likely to participate in muscle regeneration than BM cells;***, $p < .001$. (c): Quantitative real time-PCR for Pax7 expression revealed that mutant muscles receiving AFS cells (n=3) expressed higher levels of Pax7 than untreated (n=3) and BM (n=3) animals; *, $p < .05$. (d): Fifteen months after transplantation, histological and immunofluorescence analyses revealed a more sustained, long-term engraftment of AFS cells, relative to BM cells. Scale bar =100 um. (e): Graphs of raw numbers (number of fibers per area [N/A]; each letter on the x-axis is an individual muscle evaluated in three representative high-power fields) and percentages of central nucleated and GFP+ fibers (black) in untreated (blue), BM (red), and AFS-treated (green) mice at 1 and 15 months after transplantation; ***, $p < .001$.

Given the few number of freshly isolated AFS cells it was important to evaluate if their myogenic potential was maintened after a cell culture expansion. After 2 weeks of culture on a fibroblast feeder layer (Figure 13) cells grown about two folds (data not shown). Mouse AFS cells were then sorted for the dual expression of GFP and c-kit (89.50%±6.27) and transplanted in 3-months-old HSA-Cre, $Smn^{F7/F7}$ mice. Likewise to what had been observed with the freshly isolated AFS cells, one month after treatment the muscles displayed a normal phenotype: fewer than 1% of the fibres were centrally nucleated and 21.01%±3.57 were GFP^+ (Figure 13). Multiple skeletal muscle engraftments in the transplanted animals were also confirmed by RT-PCR analyses of GFP expression (Figure 13d). Twenty percent of the fibres were of donor origin, a remarkable result, given that (i) some adult stem and progenitor cell populations have proven difficult to expand in culture and (ii) skeletal muscle satellite cells multiply rapidly in culture but show diminished regenerative capacity when transplanted *in vivo* [174]. Nevertheless, 20% was significantly lower than the value achieved in animals having received freshly isolated cells. Further work is required to improve the expansion culture conditions in order to better maintain their myogenic potential. In conclusion, these data indicate that AFS cells have a myogenic potential.

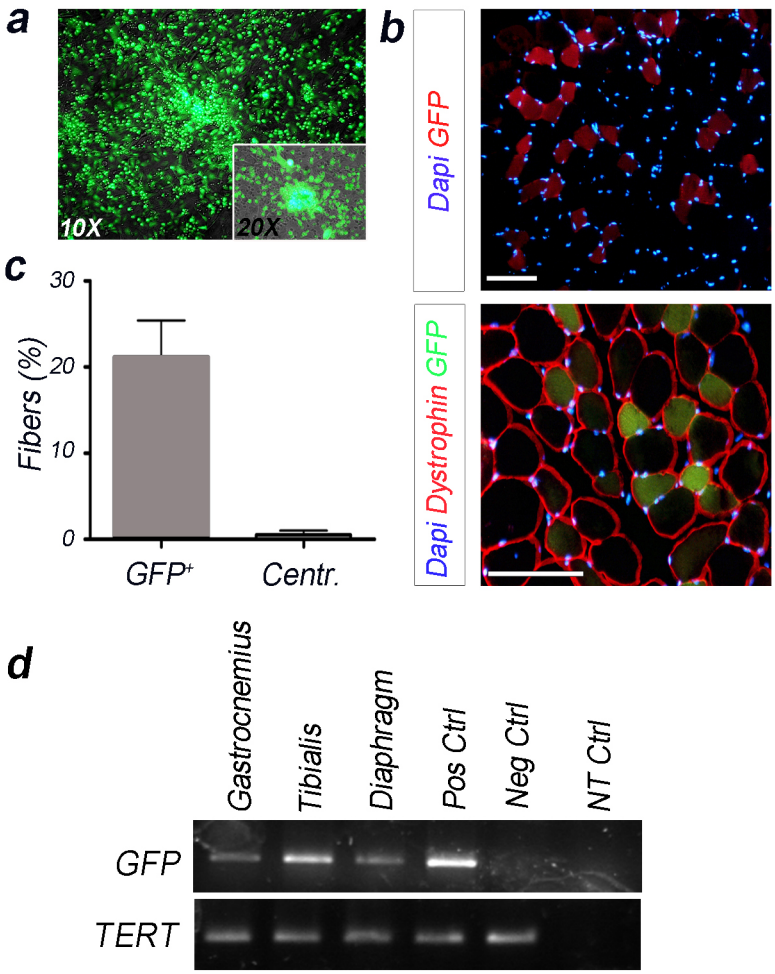


Figure 13: **Transplantation of expanded AFS cells generates skeletal muscle tissue.** (a) After 15 days of culture, AFS cells GFP$^+$/c-kit$^+$ were sorted and injected in HSA-Cre, Smn$^{F7/F7}$ mice. GFP$^+$ AFS cells were passaged twice on a mouse embryonic fibroblast feeder layer. Magnification 10X and 20X. (b) Immunofluorescence with anti-GFP and anti-dystrophin antibodies: 1 month after transplantation, the muscles of treated HSA-Cre, Smn$^{F7/F7}$ mice displayed a considerable number of GFP$^+$ fibres and a normal levels of dystrophin expression. Scale bar=100 µm. (c) Percentage of GFP$^+$ in cultured AFS-treated animals. (d) PCR analyses of gastrocnemius, TA and diaphragm muscles isolated from mice transplanted with cultured AFS cells. First lane: amplification of the GFP gene (307 bp). Second lane: amplification of genomic TERT (132 bp) to confirm the quality of the extracted DNA. For each PCR, the positive control (Pos Ctrl) for GFP amplification was the genomic DNA extracted from GFP$^+$ AFS cells; NTC=no template control.

3. Are mouse AFS cells PGC?

Moschidou et al. demonstrated that human AFS cells obtained from the first and second trimester can be reprogrammed *in vitro* when cultured under particular culture condition. Human AFS cultivated following the above mentioned method expressed markers typical of ES (like OCT4, SOX2, NANOG, SSEA3, SSEA4, TRA1-81, TRA1-60) and EG cells (FRAGILIS, TNAP, NANOS, STELLA, BLIMP, VASA, DAZL), and are able to form teratoma when injected into immunocompromised mice, and embryod bodies *in vitro*, as conseguence this cells can be defined pluripotent. Similarly PGC are not pluripotent *per se*, but following stimulation with particular cytokines they can be reprogrammed into pluripotent EG cells.

To investigate whether mouse AFS cells could be PGC, the following procedures have been assessed:

1. Cultivation of the cells using the same protcol established for the reprogramming of PGC into EG cells.
2. Use of two mouse models as PGC markers.

Regarding the first point two protocols, respectively described in [28] and [31], have been followed for the culture of mouse AFS cells. Following the first protocol, mouse AFS cells were obtained at different embryonic stages (10.5, 11.5 and 12.5) from wild type C57BL6/J fetuses, and seeded on a feeder layer of STO (a fibroblast cell line, genetically modified to express the membrane-bound form of stem cell factor, SCF) mitotically inactivated, in a medium supplemented with LIF and b-FGF. Cells were left to growth in this condition for more than 10 days (time at which normally EG colonies start to appear after the reprogramming of PGC). Mouse AFS cells never formed EG colonies when cultivated in this condition, and when analyzed for the expression of the plurypotency markers (Oct4 and Sox2) by immunofluorescence, they were negative for these markers (data not shown, n=5). This result highlighted that no reprogramming process was on going. The second protocol [31] has also been tested for the reprogramming of mouse AFS. Mouse AFS cells were obtained from Oct4-GFP fetuses (with a mixed background CD1/129) at E10.5 and E11.5. This mouse model

gives the possibility of monitoring and identify cells GFP positive, signal of the endogenous expression of the gene Oct4. As positive control genital ridges (GRs) obtained from the same fetuses have been used. A feeder layer of mitotically inactivated Sl^4-m220 (that like STO are a fibroblast cell line, genetically modified to express the membrane-bound form of stem cell factor, SCF) has been used to seed mouse AFS and GRs; for the first two days in culture they were feeded with PGC medium supplemented with LIF and FGF-2, and from the third day and on with the N2B27 2iLIF medium (experiments n=5). While the reprogramming protocol worked for GRs seeded, in fact they formed EG colonies that were GFP positive (Figure 14), no colonies appeared in the culture of mouse AFS cells.

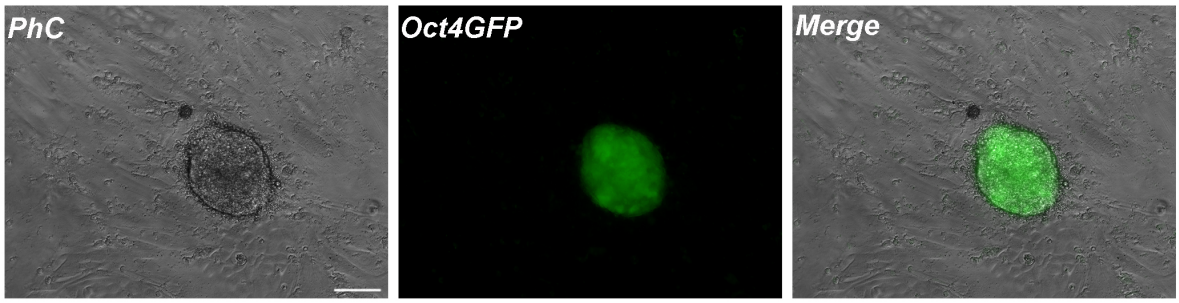


Figure 14: **Mouse EG cells obtained from Oct4-GFP GRs in N2B27 2i-LIF condition.** Phase contrast and fluorescence images of Oct4-GFP EG cells obtained after 11 days in culture of PGC obtained from GRs at E11.5, cultured on a Sl^4-m220 feeder layer. Scale bar=100um.

Following the second strategy, to deeper investigate the possible PGC nature of AFS cells two mouse models has been used: the Oct4-GFP and TNAP-Cre. The Oct4-GFP mice is characterised by the insertion of a gene reporter, the Green Fluorescence Protein (GFP), into the Oct4-genomic fragment of 18kb, this encompass the Oct-4 gene and its 5'- and 3'-flanking sequences. The transgene reproduced the endogenous expression pattern of Oct-4 in embryos and in the germ cell lineage; so the Oct4 expression is restricted only in the PGC from E10.5 until E13.5 (for female, while for male germ cells the expression of endogenous Oct-4 continue even after they are mitotically arrested), [175]

Alkaline phosphatase (AP) is one of the first marker that identifies PGC in the allantois during the mouse embryo development. In the mouse model TNAP-Cre the Cre

recombinase is knocked into the locus of the Tissue Non-Specific Alkaline Phosphatase, TNAP gene. In these models mouse AFS cells were isolated and analized by flow cytometry for the expression of Oct4 and TNAP. Regarding the Oct4-GFP model the AF was collected from pregnant females (obtained from mating of males Oct4-GFP CD1 x females 129 or males 129 x females Oct4-GFP CD1), and analyzed to see if in the c-kit^{+} cells of the AF there were Oct4GFP+ cells. The AF was collected from different embryonic stages (E10.5 experiment n=1, E11.5 experiment n=1, E12.5 experiment s n=4, E13.5 experiment n=2) the linegage depleted cells were incubated with c-kit antibody and a viability probe to exclude the death cells, analyzed by flow cytometry. In all the samples studied there weren't signals for the GFP, as shown in the figure 15, while the GFP signal was present in the positive control, constituted by GRs obtained from the same fetuses from which AF was collected. For each analyzed sample the threshold of GFP positivity was defined after the comparision between GRs and AFS cells obtained from Oct4-GFP positive and negative fetuses. In figure 15 are showed results obtained only from Oct4-GFP positive fetuses.

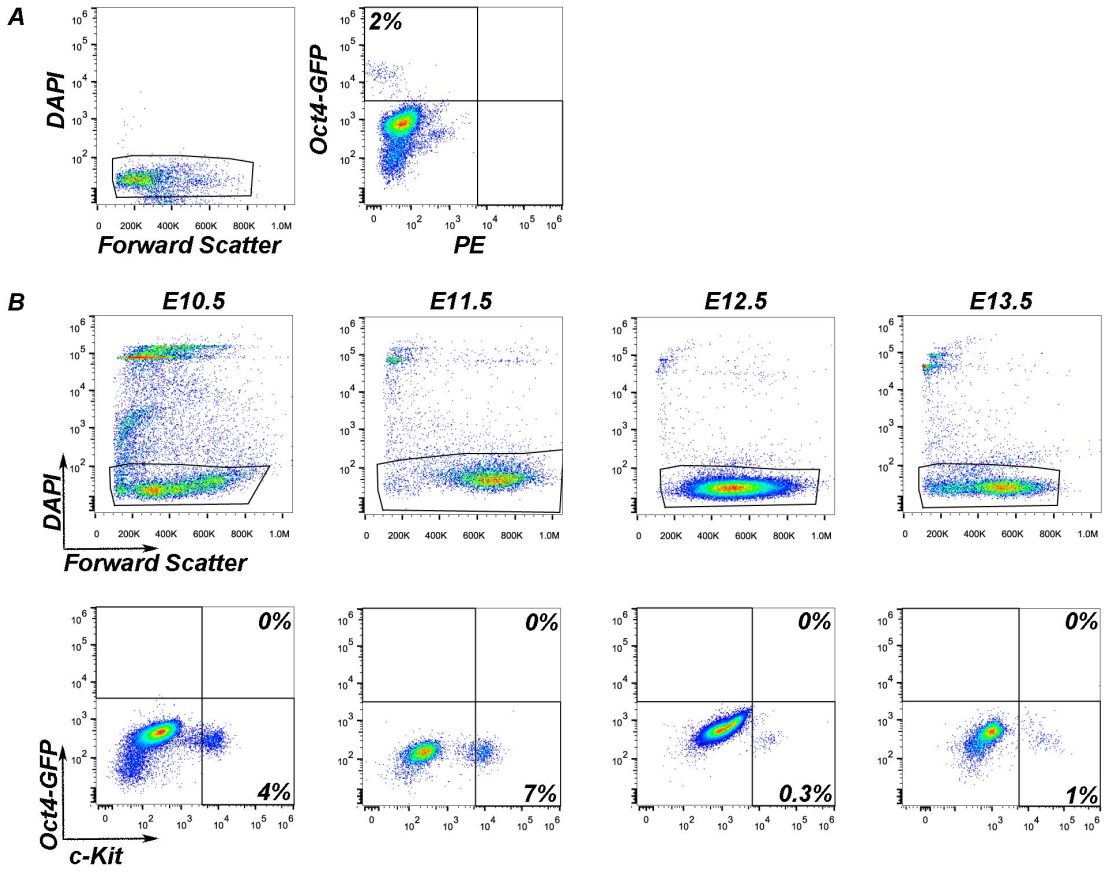


Figure 15: **Flow Cytometry analisys.** (A) Representative Dot Plots of cells obtained from GRs collected from Oct4-GFP^{+} fetuses showed that in the viable cells the expression of Oct4 is around 2% (B) mouse AF cells collected from Oct4-GFP positive fetuses were lineage depleted and stained with antibody specific for c-kit markers and for the viability probe DAPI. In all the embryonic stages analyzed, no signal for GFP was detected.

Regarding the TNAP-Cre mouse model the AF was collected from fetuses obtained from mating TNAP-Cre males with different females (Z/Red or Z/eGFP, in which there is the expression of lacZ before Cre-mediated excision and the Red or enhanced GFP genes after Cre excision; or Tomato in which after Cre-mediated excision there is only the Tomato expression), and analyzed at two embryonic stages (E10.5 and E13). Unfortunately TNAP-Cre mouse model turned out not specific for the purpose, because the fluorescence signal in the fetuses was spread in the whole body and not present only in the PGC (Figure 16), indicating that the Cre-mediated excision was happened in different tissues.

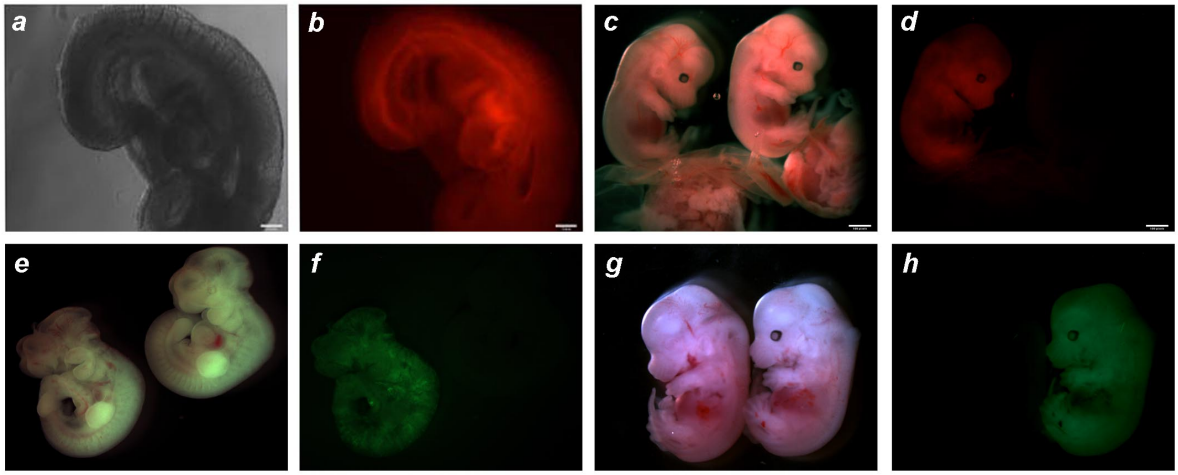


Figure 16: **TNAP expression.** (a,b) A whole-mount E8.5 double transgenic (TNAP-Cre-Tomato) embryo showing the Tomato signal in the whole body (c,d) A whole-mount E13 double transgenic (TNAP-Cre-Z/Red) embryos showing the Red signal in the whole body. Here there is also the comparison with a wild type embryo (e,f,g,h) A whole-mount E10.5 and E13 double transgenic (TNAP-Cre-Z/eGFP) embryos showing the GFP signal in the whole body.

Results obtained from these experiments told us that mouse AFS cells cannot be re-programmed *in vitro* like PGCs did, and they don't express Oct4.

4. Induction of pluripotency of mouse AFS cells

To explore the reprogramming of mouse AFS cells a protocol that involve the use of a non-viral method, the PB system, has been established. PB transposition is host-factor independent, and has recently been demonstrated to be functional in various human and mouse cell lines [176,177,178,179]. The PB transposon/transposase system requires only the inverted terminal repeats flanking a transgene and transient expression of the transposase enzyme to catalyse insertion or excision events [180]. For the induction to the pluripotency the four Yamanaka's factors (Oct4, Klf4, c-Myc and Sox2) have been used, they were toghether in an ORFs linked with 2A peptide sequences into the PB-TET transposon plasmid (called PB-TET-OKMS), under the transcriptional control of the $tetO_2$ tetracycline/doxycycline inducible promoter. All were linked to the gene of a cherry fluorescent protein through an IRES sequence, to allow monitoring the tightness of doxycycline regulation and later demonstration of the reprogrammed cells capacity for exogenous-factor-independent maintenance. The reverse tetracycline transactivator (rtTA) protein, necessary for the activation of the expression control by doxycycline, was provided also by another transposon plasmid. AF were collected from fetuses obtained from Oct4-GFP mice, used to monitor the expression of the endogenous Oct4 in reprogrammed cells. Mouse AFS were transfected with circular PB-TET-OKMS and rtTA plasmids in conjunction with the PB transposase expression plasmid (mPBase). In two separate experiments, performed according to the protocol described in figure 17, mouse AFS cells culture were transfected with Oct4, Klf4, c-Myc and Sox2. Mouse AFS underwent ES-cell-like colony formation, which resulted in the derivation of self-renewing cell lines displaying key characteristics of reprogramming.

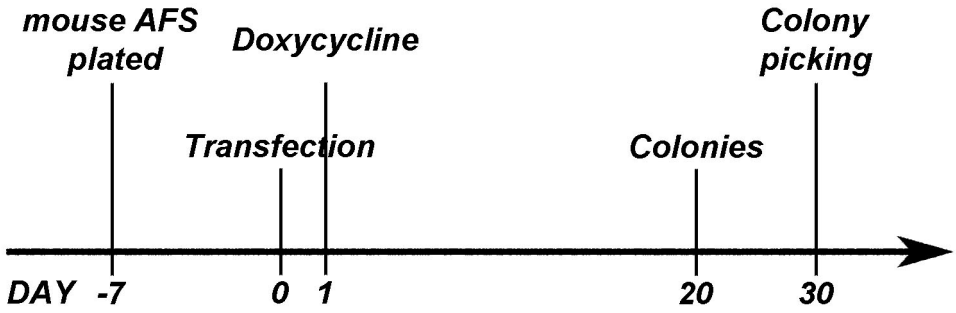


Figure 17: **Reprogramming protocol of mouse AFS cells.** Mouse AFS cells were plated on a feeder layer of mitotically inactivated MEF in ES medium, and transfected using fugene (day 0) with PB-TET-OKMS, PB-rtTA and mPBase. Expression of 4 factors was achieved by adding doxycycline in culture (day 1), 24 hours after the transfection. Colonies emerged 20 days post-transfection and they were picked on day 30 and transferred to feeder layers in ES Medium with/without doxycycline.

The first colonies appeared 20 days after doxycycline induction, and they were picked after 30 days. The standard concentration for the doxocyclyne was 1.5 ug/ml, as previously described [120]. Around twenty PB-TET-OKMS-induced colonies were picked from mouse AFS induction fields and passaged on inactivated fibroblast feeder layers. Surviving clones were maintained in doxycycline during establishment, until found to be doxycycline independent in replicate wells as it is shown in figure 18. The clones tested passed the alkaline phosphatase staining criterion, and they were positive for the cell-surface marker SSEA1 and nuclear-localized Nanog protein (Figure 19).

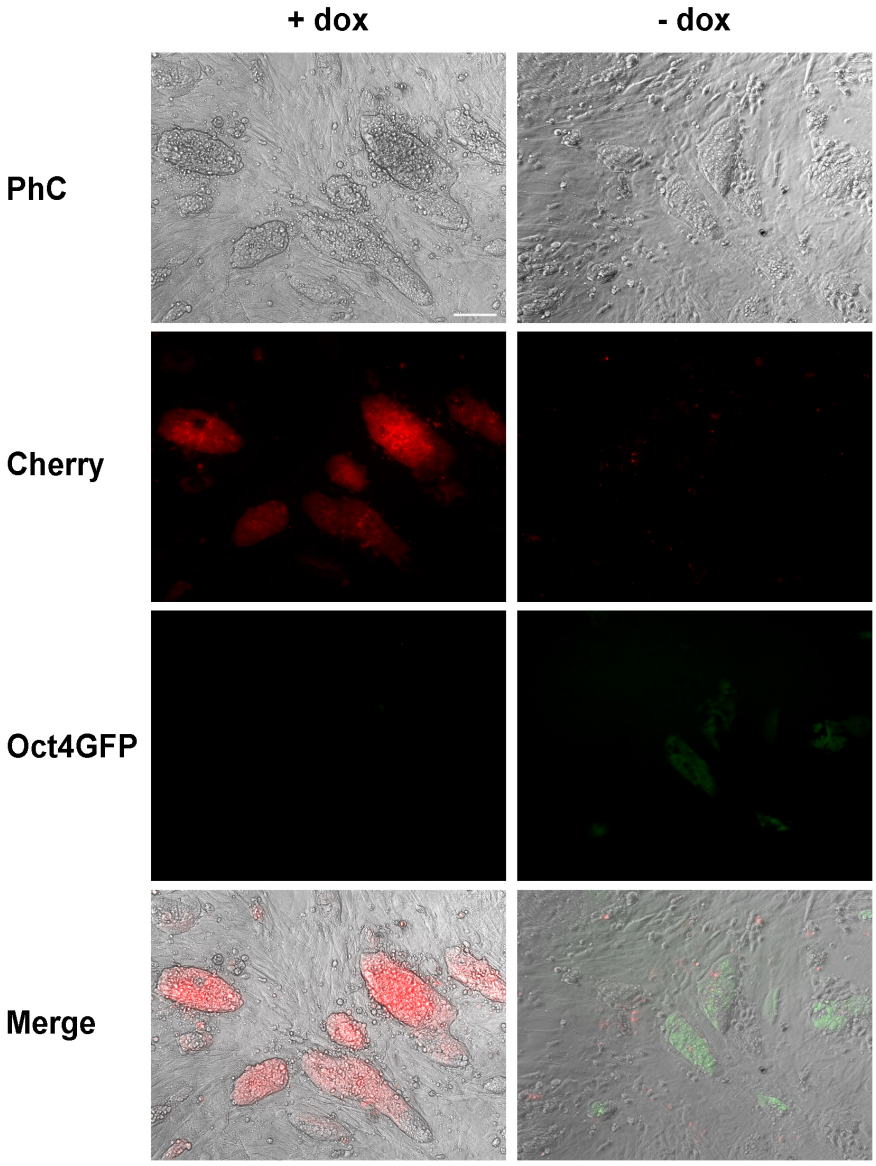


Figure 18: **Detection of the exogenous factors and endogenous Oct4 in reprogrammed clone.** PB-TET-reprogrammed clones were screened for cherry and Oct4 expression to determine general transgene activity in the presence and absence of doxycycline. Scale bar= 100um.

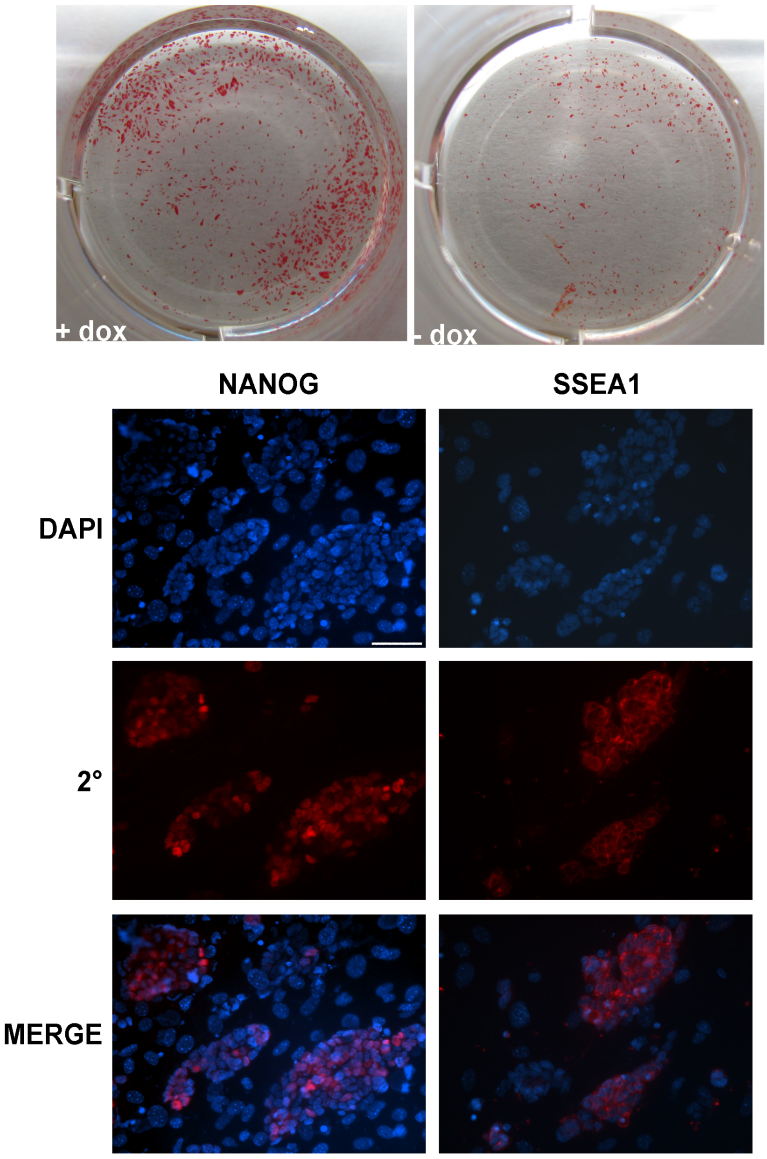


Figure 19: **Cell lines generated by PB-mediated factor transposition.** Stable doxycycline-independent cell lines activate alkaline phosphatase (AP), SSEA1 and Nanog. Scale bars, 100um.

VII. Discussion

Experiments accomplished in this thesis have confirmed that a population of c-kit^{+} cells is present in the mouse amniotic fluid, like described also in [64] and [62], and their number change during the course of gestation. A slight difference regarding the number of c-kit^{+} cells present in the amniotic fluid has been highlited between this data and data obtained from [64], this probably was due to the different lineage depletion cocktail of antibodies used; in Ditadi at al., it was consistituted from: CD3, CD4, CD8, CD19, B220, Gr1, Mac1, Ter119, and NK1.1, while the one used here was composed from: CD5, CD45R(B220), CD11b, Anti-Gr1 (Ly-6G/C), 7-4 (Ly-6B), Ter119. Flow cytometer analysis have highlighetd that mouse AFS cells expressed markers of mesenchymal stem cells (CD90, CD105), hematopietic cells (CD45, CD34, Sca1) and also endothelial cells (Flk1, CD31). Two distinct populations have been detected in the amniotic fluid, ditsinguishable for the intensity of expression of the marker c-kit, for this reason they were called c-kit^{high} and c-kit^{low}. They differed also for the expression of some other evaluated markers, in fact c-kit^{high} expressed only CD45, CD90 and CD44 while c-kit^{low} expressed all the markers analyzed. Their hematopietic potential has been studied, but no differences showed up, in fact under appropriate differentiation conditions c-kit^{high} and c-kit^{low} were able to generate all the blood lineages (ie, myeloid and erythroid colonies). *In vitro* experiments didn't highlight differences between the two populations, so *in vivo* experiments will be necessary to evaluate their potential.

Mouse AFS cells were analyzed for the gene expression of the pluripotency markers c-Myc, Klf4, Oct4, Sox2 and Nanog at population level and also in single cells PCR. Quantitative Real Time PCR at populations level revealed that expression was very low if compare with expression of the same gene of mouse ES cells. There were no expression for the genes Nanog and Sox2 (at E11.5). Genes expressed at higher levels than mouse ES cells were c-Myc and Klf4 at E11.5. For a more detailed analysis a single cells PCR was done at different embryonic stages (10.5-14.5). It has been found that in almost all the embyonic stages evaluated there were cells that co-expressed Sox-2 and c-Myc (sm), Klf4 and c-Myc (km). Besided at the embryonic

stages 12.5 and 13.5 different cells were found to co-express more pluripotency markers (see table 2), and the embryonic stage 13.5 was the only one in which there were 5% of cells that co-express three relevant pluripotency marker (Klf4, Oct4 and Sox2). The novelty and the surplus value of the single cell approach in respect to populations analysis is represented by the fact that with this technique it is evaluated not only the expression of each considered gene, but also the co-expression of more genes in the same cell, that is closely related to the biological activity and the differentiation capacity of that cell. These data confirmed cellular heterogeneity of mouse AFS cells and suggest that at E13.5 cells are closer to mouse ES cells.

Results obtained after the injection of mouse AFS cells into the mouse model of SMA, HSA-Cre $Smn^{F7/F7}$ suggest that they have a therapeutic effect, similar to mouse BM cells, when compared one month after the transplantation. However relevant differences have been identified in a long term period, in fact 15 month post transplatation BM-treated displayed a high number of central nucleated fibers (39.90% ± 17.68%) and consistent infiltration of interstitial tissue between the myofibers, a situation that differed greatly from the one observed in AFS-treated mice. Moreover, at this stage (15 months pt), no GFP^+ fibers were found in BM-treated animals. This finding highlights the sustained effect of AFS cells transplantation. These long-term effect suggest that mouse AFS cells, differently to mouse BM cells, could integrate into the muscle stem cells niche, but more experiment will needed to evaluate the hypothesis. Expanded AFS cells intravenously injected demonstrated the maintenance of the cells's regenerative properties after culture. Twenty percent of the fibers was of donor origin, a remarkable result, given that (a) some adult stem and progenitor cells have proven difficult to expand in culture and (b) skeletal muscle SCs multiply rapidly in culture but show diminished regenerative capacity when transplanted *in vivo* [174]. Nevertheless, 20% was lower than the value achieved in animals having received freshly isolated cells. Further work is required to assess the relationship between myogenic potential and expansion conditions.

Potential sources of AFS cells in the developing fetus are diverse [181]. CD117 (c-Kit), the surface marker used for immunoselection of AFS cells, plays an important

role in gametogenesis, melanogenesis and hematopoiesis [182,183]. This knowledge together with the recent demonstration that human AFS cells, obtained from first and mid trimester, can be reprogrammed to pluripotency *in vitro*, without the need to force the expression of Yamanaka's factor, but only if stimulated with VPA [71,72], suggested the idea that AFS cells could be PGC. PGC are not pluripotent, but they can achieved pluripotency *in vitro*, and became EG cells, if stimulated with particular cytokines. To sustain this hypothesis it has been observed that during the mouse embryonic development, the migration of PGCs is time-correlated with the formation of the amnion [167]. Mouse AFS cells have been cultivated following the protocol used to reprogramm PGC into EG cells [28,31]. Mouse AFS cells did not form EG cells, while single cells obtained from GRs, reprogrammed under the same condition. Two mouse models have been used for investigation of this idea: Oct4-GFP and TNAP-Cre. Lomeli H at al., evaluated the *in vivo* specificity of excision achieved by the TNAP-Cre mouse lines by crossing them with the double-reporter line, Z/AP. The Z/AP transgene expresses lacZ before Cre-mediated excision and the heat-resistant human alkaline phosphatase (hAP) gene after Cre excision. Double transgenic TNAP-Cre/Z/AP embryos showed the initial hAP activity was specific to the PGCs at E10.5. After midgestation, however, it was also expressed in the labyrinthine region of the placenta, the intestine and the neural tube. A detailed analysis of E13 embryos from crosses between TNAP-Cre males and Z/AP females showed 8 out of 16 embryos were positive exclusively in PGCs. [184] The same TNAP-Cre mouse line has been crossed with different report lines: Z/eGFP, Z/Red and Tomato. All the double-transegnic embryos analized showed a Cre exicision not restricted to PGC,for this reason no conclusion can be obtained from the use of the TNAP-Cre line, as it showed up to be unspecific (see figure 16). The analysis of the Oct4-GFP mice demonstrated that no Oct4 positive cells were present in the AF. The results from the reprogramming experiments, together with the ones obtained from the Oct4-GFP line tell us that mouse AFS cells are not PGC. However it is important to remind that Oct4-GFP mice are not lineage tracking model, so if mouse AFS cells, during the embryo development, go through the germ cells lineage it is still unknown. More experiments will be necessary to answer at the origin question and probably it will be necessary to investigate not only the germ cells origin hypothesis.

Since their discovery in 2006 the iPS cells have opened great expectation for the treatment and study of diseases. So far in different works they have been used in mice for the treatment of some disease, like for example hemophilia A [134] and sickle cell anemia [133]. iPS cells have also been established from patients with adenosine deaminase deficiency, Schwachman-Bodian-Diamond syndrome, Gaucher disease, Duchenne and Becker muscular dystrophy, Parkinson disease, Huntington disease, type 1 diabetes mellitus, Down syndrome, Lesch-Nyhan syndrome, amyotrophic lateral sclerosis, spinal muscular atrophy, and Fanconi anemia [118,135,136,137,138]. The recent finding regarding the low immunogenicity of transplanted cells differentiated from iPSCs [185] make them a more promising research tool. Considering all the congenital diseases that can be detected in the fetus through the prenatal diagnosis, cells obtained from the amniotic fluid can be a good source to obtain iPS that could be used both for the treatment and the study of the disease. The method used to obtain the induction of pluripotency is relevant for their safety. The PB system is a non-viral method suitable for the reprogramming, it allow the production of xeno-free iPS cells contrary to current viral production protocols that use xenobiotic conditions. The accurate transgene removal through transposase expression has been demonstrated in various cell lines [178,179,180]. Here we have showed that the PB system is a suitable method for the reprogramming of mouse AFS cells. These are only preliminary results and more experiments will be necessary for complete the characterisation of the cells. The next step will be to use the same protocol used here to obtain iPS from human AFS cells obtained from healthy and diseased fetuses.

The "perfect" cell source for regenerative medicine should be derived from ethically acceptable sources, should have the potential to be maintained in culture indefinitely, should be inducible to differentiate into mature cell from all the tissues, and should be safe. Several cells type have been studied that might respond (totally or in part) to those requirements. In theory, a bank of 100.000 amniotic fluid specimens could potentially supply 99% of the US population with a perfect match for transplantation [42] thus making AFS a truly promising source of cells for regenerative medicine therapies.

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