Chapter 5

Human amniotic fluid stem cells as a source of hepatic and endothelial progenitors for \textit{in vivo} cell therapy
ABSTRACT

Human amniotic fluid has been utilized in prenatal diagnosis for over 70 years. It has proven to be a safe, reliable, and simple screening tool for a wide variety of developmental and genetic diseases. There is now evidence that amniotic fluid may have more utility than only as a diagnostic tool, and it may be a source of a powerful therapy for a multitude of congenital and adult disorders. In this chapter, we describe the development of the amniotic fluid in the human, and the cells that are found within the amniotic fluid. We will further describe previous research suggesting that amniotic fluid have progenitor cells, and the recent data from our lab demonstrating a rare population of stem cells from amniotic fluid, termed amniotic fluid stem (AFS) cells that proliferate readily in culture and differentiate into various cell types. We will also confirm that these cells don’t form teratomas and show their potential to integrate in regenerating tissues in vivo. It is possible that in the near future, we will see the development of therapies utilizing progenitor cells isolated from amniotic fluid for the treatment of newborns with congenital malformations as well as in adults using cryopreserved amniotic fluid stem cells.
5.1 INTRODUCTION

Amniotic fluid derived stem (AFS) cells can be obtained from a small amount of an amniotic fluid specimen during amniocentesis, a procedure that is frequently performed in many of the pregnancies in which exists a risk of congenital abnormality of the fetus. Analysis of cell cultures from this source, provide evidence that they may represent a new source for the isolation of cells with the potency to differentiate into different cell types, suggesting a new source of cells for research and regenerative medicine.

5.1.1 DEVELOPMENT AND CHARACTERIZATION OF AMNIOTIC FLUID

Amniotic Fluid in Developmental Biology

Embryonic and fetal cells derived from all three germ layers and extraembryonic tissues have long been identified in the amniotic fluid\textsuperscript{1-3}. Regardless of the fact that the specific origins of many subsets of these cell types still remain to be determined, their origin in the amniotic fluid is closely tied with the developmental processes unfolding \textit{in utero} at a particular gestational age. It is not surprising that the profile of the cellular component of the amniotic fluid varies with gestational age\textsuperscript{4}.

A major milestone in early post implantation development is gastrulation. At about embryonic day 15 (E15), gastrulation begins in the posterior region of the human embryo. Pluripotent epiblast cells are allocated to the three primary germ layers of the embryo (ectoderm, mesoderm, and endoderm) and germ cells, which are the progenitors of all the tissue lineages as well as the extraembryonic mesoderm of the yolk sac, amnion, and allantois\textsuperscript{5}. The latter forms the umbilical cord as well as the mesenchymal part of the labyrinthine layer in the mature chorio-allantoic placenta. The final positions of the fetal membranes result from the process of embryonic folding or turning, which occurs around human E21 of gestation and ‘‘pulls’’ the amnion and yolk sac around the embryo. The amniotic cavity pushes in also at the cranial and caudal ends of the embryonic disc, thus increasing the degree of longitudinal folding at the head and tail folds. The amniotic cavity also pinches the connection of the yolk sac and gut to form the narrowed communication of the vitello-intestinal (or vitelline) duct\textsuperscript{6}.
Once formed, the amniotic sac is a tough but thin transparent pair of membranes that holds a developing embryo (and later fetus) until shortly before birth. The inner membrane, the amnion, contains the amniotic fluid and the fetus. The outer membrane, the chorion, contains the amnion and is part of the placenta. With all this developmental processes unfolding, it is not surprising that a wide variety of different origins has been suggested for the mixture of cells within amniotic fluid.

**Amniotic fluid Secretion and Dynamics**

In humans, as mentioned above, the amniotic cavity starts to form immediately prior to gastrulation (E8-9). Once the lining of the amnion is completed, the amniotic cavity fills with amniotic fluid secreted by the cells in the amniotic membrane. This fluid serves as a shock absorber for the developing embryo while prevents its desiccation. It also ensures symmetrical structure development and growth maintaining consistent pressure and temperature. The fluid permits freedom of fetal movement, important for musculoskeletal development and blood flow.

In the first half of gestation, most of the amniotic fluid results of active sodium and chloride transport across the amniotic membrane and fetal skin, with concomitant passive movement of water. In the second half of gestation, most of the fluid comes from fetal urine. An additional major source of amniotic fluid is secretion from the respiratory tract. Fetal swallowing and gastrointestinal tract excretions, while not voluminous, also play a role in amniotic fluid composition.

These fluid dynamics are responsible for the production and turnover of the amniotic fluid and are also thought to be important in determining the cell types present in the amniotic cavity. Still, much remains to be clarified about the ontogeny of many subsets of amniocytes at any gestational age.

**Cell Populations in Amniotic Fluid**

Amniotic fluid specimens have been long used as a source of human cells in prenatal sex determination and diagnosis of genetic disorders. These cells also constituted early on a precious
source of human cells for the purpose of biological investigation\textsuperscript{17-19}. Several morphologic cell types can be found in \textit{in vitro} cultures of amniotic fluid. These cells have been classified into three generic different classes, according to their clonal growth and morphology. The most frequent are the AF-Type of colonies which are constituted by small cells with densely staining nuclei and only subtly distinct from “classical” fibroblasts. With the greatest \textit{in vitro} growth potential of all colony-forming cells are the F-Type colony cells that consist in fibroblast-like cells. The other clonal cell population usually identified is the E-Type colony cells that have large polygonal shape with smooth margins and grow in intimate contact with each other. The morphologies found on some of these cultured cells (e.g. epithelioid, fibroblastoid, etc) also confirms the putative origin attributed to cells in amniotic fluid as cells with origin in the fetal amnion, skin, urinary, respiratory, and gastrointestinal tracts that can be shed into the amniotic cavity and fluid\textsuperscript{1-3}.

Despite the fact that cell clonal populations were identified in amniotic fluid culture quite early, it was only in 1993 that small, nucleated, round cells identified as hematopoietic progenitor cells were found as the first reported progenitor population present in amniotic fluid\textsuperscript{20}. A study in 1996 was the first to suggest the possibility of multi-lineage potential of non-hematopoietic cells present in the amniotic fluid, by showing myogenic differentiation of amniocytes\textsuperscript{21}.

The presence of mesenchymal cells in the amniotic fluid has also been proposed for decades\textsuperscript{22, 23}. Nonetheless, the differentiation potential of mesenchymal amniocytes started to be unveiled only very recently\textsuperscript{24-27}. In 2001, Kaviani \textit{et al} reported that only 2 milliliters of amniotic fluid can give about 20,000 cells, 80 % of which are viable\textsuperscript{28, 29}. Further, Tsai \textit{et al} reported that amniotic fluid cells takes 20 to 24 hours to double the number of cells collected, which is faster than umbilical cord stem cells (28 to 30 hours) and bone marrow stem cells (over 30 hours)\textsuperscript{30}. The proliferation rate of the cells is a very important feature for using the cells for urgent medical conditions. The isolation success rate for amniotic fluid cells is close to 100 percent, whereas, scientists have only been able to isolate and differentiate on average just 30 percent of mesenchymal stem cells (MSC) extracted from a child’s umbilical cord shortly after birth\textsuperscript{30, 31}. Furthermore, extracting the cells from one’s own amniotic fluid bypasses the problems associated with a technique called donor-recipient HLA matching, which involves transplanting cells\textsuperscript{32}.  

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Similarly, the presence of embryonic stem cells in the amniotic fluid was suggested only in the last couple of years\textsuperscript{32-34}. Prusa \textit{et al.} demonstrated Oct-4 expressing cells and neurogenic cells in human amniotic fluid\textsuperscript{35-37}. In 2001, our lab initially reported that we could isolate a subpopulation in human amniotic fluid that could differentiate into multiple lineages\textsuperscript{38}. In January 2007, we reported the isolation of clonal lines of amniotic fluid stem (hAFS) cells that express embryonic and adult stem cell surface markers\textsuperscript{39}.

Human amniotic epithelial cells (i.e. cells isolated from the Amnion derived from term placentas, not from the amniotic fluid) have also shown multipotent potential to differentiate to all three germ layers - endoderm (liver, pancreas), mesoderm (cardiomyocyte), and ectoderm (neural and glial cells) \textit{in vitro}\textsuperscript{40-43}.

The identification of several stem/progenitor cell populations in amniotic fluid, suggested that assessment of expression of specific markers is vital to fully elucidate the presence of certain stem/progenitor cell populations and number of these cells in amniotic fluid. Flow cytometric analysis of cells obtained from primary cultured cells without expansion of amniocentesis specimens, revealed low cell numbers for most of the stem/progenitor cell markers analyzed\textsuperscript{45} (Table 5.1).

\begin{table}[h]
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\begin{tabular}{|l|l|}
\hline
Marker & Percentage Positive \\
\hline
CD117 (c-Kit) & 0.9±0.2 \\
CD34 & 2.8±0.3 \\
CD105 & 19.5±1.1 \\
CD133 & 0.9±0.2 \\
\hline
\end{tabular}
\caption{Flow cytometric analysis of cells obtained from primary cultured cells without expansion of amniocentesis specimens. Values are mean ± s.e.m. for five independent amniotic fluid specimens.}
\end{table}
This result suggested that sorting of specific stem/progenitor cell populations from amniotic fluid could be made possible using an appropriate method and/or technology. The use of anti c-kit isolation of AFS cells exposed a new population of clonal stem cells for use in human tissue engineering and regenerative medicine.

5.1.2 AFS ISOLATION, CHARACTERIZATION AND DIFFERENTIATION

Isolation of AFS cells
AFS cells are separated from the amniotic liquid by centrifugation. AFS cells are allowed to proliferate in vitro in culture medium (consisting of modified α-Modified Earls Medium, 18 % Chang Medium B, 2 % Chang C with 15 % embryonic stem cell certified fetal bovine serum, antibiotics and L-glutamine). In culture, the progenitor cells maintain a round shape for one week post separation when cultured in non-treated culture dishes. In this state, they demonstrate a very low proliferative capability. After the first week the cells begin to adhere to the plate and change their morphology, becoming more elongated and proliferating more rapidly, with a need for passage every 48-72 hours. No feeder layers are required either for maintenance or expansion. Once the initial cells are established in culture, a pluripotent subpopulation of progenitor cells can be isolated through positive selection for cells expressing the membrane receptor c-kit. We employed immunoselection with magnetic microspheres to isolate the c-Kit – positive population from many amniocentesis specimens.

C-kit receptor
C-kit is a protoncogene that encodes a 14.5-kD transmembrane receptor, p145 (CD117 or KIT, or stem cell factor receptor). This receptor belongs to the class III of receptor tyrosine kinase family. It was first identified as a viral transforming gene (v-kit), an oncogene derived from the feline retrovirus HZ4-FeSV, being c-kit its normal cellular homologue. Due to its feline origin, that this protoncogene was named as c-kit, for kitten. The ligand for this receptor is the Stem Cell Factor protein, also known as Mast Cell Growth Factor or Steel Factor. KIT plays a key role during fetal development, and its expression is constitutively maintained in hematopoietic stem cells, mast cells, intraepithelial lymphocytes, germ cells, melanocytes, and interstitial cells of Cajal. It is associated with immature stages of hematopoiesis, melanogenesis,
osteoclast and Langerhans cells differentiation, which lose the antigen during differentiation into more mature cells\textsuperscript{48-51}.

Several mutations were identified in W mutant mice resulting in anemia, lack of mast cells, pigmentation defects and infertility, what emphasizes its key role in fetal development and as a growth factor receptor\textsuperscript{52, 53}. Altered c-kit levels also occur in a variety of malignancies and cancers, like leukemias, lymphomas, small cell lung cancer and melanoma, just to mention a few\textsuperscript{46, 53}.

Due to the critical role of c-kit in proliferation and survival of several stem/progenitor cell populations, it was sought as a good strategy to isolate stem/progenitor cells from amniotic fluid cultured cells.

**AFS Cell Culture and Characterization**

About 0.8 % to 1.4 % of cells present in amniotic fluid and placenta have been shown to be c-kit positive. Of these, over 90 % expressed the transcription factor Oct4 which has been associated with maintenance of the undifferentiated state and the pluripotency of embryonic stem cells\textsuperscript{54}. The progenitor cells derived show a high self-renewal capacity with >300 population doublings, far exceeding Hayflick’s limit. The doubling time of the undifferentiated cells is noted to be 36 hours with little variation with passages.

These cells have been shown to maintain a normal karyotype at late passages and have normal G1 and G2 cell cycle checkpoints. They demonstrate telomere length conservation while in the undifferentiated state as well as telomerase activity even in late passages\textsuperscript{55}. Analysis of surface markers shows that progenitor cells from amniotic fluid express human embryonic stage specific marker SSEA4, and the stem cell marker OCT4, and did not express SSEA1, SSEA3, CD4, CD8, CD34, CD133, C-MET, ABCG2, NCAM, BMP4, TRA1-60, and TRA1-81. These characteristics demonstrate that AFS cells express some of some key markers of embryonic stem cell phenotype, but not the full complement of markers expressed by embryonic stem cells. This may indicate that the amniotic cells are not quite as primitive as embryonic cells, yet are more primitive than most adult stem cells. Importantly, AFS cells do not form teratomas \textit{in vivo} when
implanted in immunodeficient mice. Lastly, cells, when expanded from a single cell, maintained similar properties in growth and potential as the original mixed population of the progenitor cells.

**AFS Cell Differentiation**

AFS cells have been shown to be pluripotent and differentiate into osteogenic, adipogenic, myogenic, neurogenic, endothelial, and hepatic phenotypes in vitro. Each differentiation has been performed through proof of phenotypic and biochemical changes consistent with the differentiated tissue type of interest.

**Adipocytes**

To promote adipogenic differentiation, AFS cells can be induced in dexamethasone, 3-isobutyl-1-methylxanthine, insulin, and indomethacin. The cells cultured with adipogenic supplements change their morphology from elongated to round within 8 days. This coincides with the accumulation of intracellular droplets. After 16 days in culture, more than 95% of the cells have their cytoplasm filled with lipid-rich vacuoles. Adipogenic differentiation also demonstrates the expression of peroxisome proliferation-activated receptor γ2 (pparγ2), a transcription factor that regulates adipogenesis, and of lipoprotein lipase through RT-PCR analysis\(^56,57\). Expression of these genes is noted in the cells under adipogenic conditions but not in undifferentiated cells.

**Osteocytes**

Osteogenic differentiation was induced in the AFS cells with use of dexamethasone, betaglycerophosphate, and ascorbic acid-2-phosphate\(^58\). The cells maintained in this medium demonstrated phenotypic changes within 4 days with a loss of spindle shape phenotype and development of an osteoblast-like appearance with fingerlike excavations into the cytoplasm. At 16 days, the cells aggregated, showing typical lamellar bone-like structures. In terms of functionality, these differentiated cells demonstrate a major feature of osteoblasts which is to precipitate calcium. Differentiated osteoblasts from the AFS cells are able to produce alkaline phosphatase (AP) and to deposit calcium, consistent with bone differentiation. The undifferentiated cells lacked this ability. The AFS cells in osteogenic medium express specific
genes implicated in mammalian bone development which are AP, core binding factor A1 (cbfa1), and osteocalcin in a pattern consistent with the physiological analog.

**Endothelial cells**

The AFS cells can be induced to form endothelial cells (EC) by culture in endothelial basal medium on gelatin coated dishes. Full differentiation is achieved after one month in culture; however, phenotypic changes are noticed within one week of initiation of the protocol. EC differentiated AFS cells show expression of human-specific endothelial cell surface marker (P1H12), factor VIII (FVIII), VEGF Receptor-2 (KDR) and CD31 that are specific for differentiated endothelial cells. The AFS cells do not stain for endothelial specific markers. The AFS derived endothelial cells, once differentiated, are able to grow in culture and form capillary-like structures in vitro.

**Hepatocytes**

For hepatic differentiation, the AFS cells are seeded on matrigel or collagen coated dishes at different stages and cultured in the presence of hepatocyte growth factor, insulin, oncostatin M, dexamethasone, fibroblast growth factor 4, and monothioglycerol for 45 days\(^{59, 60}\). After 7 days of the differentiation process, cells exhibit morphological changes from an elongated to a cobblestone appearance. The cells show positive staining for albumin at day 45 post differentiation and also express the transcription factor HNF4α, the c-met receptor, the MDR membrane transporter, albumin, and α-fetoprotein. Hepatic differentiation induced AFS cells produced urea at a level of \(1.21 \times 10^3\) ng urea/hour/cell compared with \(5.0 \times 10^1\) ng urea/hour/cell for the control cell populations\(^61\).

**Myocytes**

Myogenic differentiation is induced in the AFS cells by culture in media containing horse serum and chick embryo extract on a thin gel coat of matrigel\(^62\). In order to initiate differentiation, the cells are incubated in the presence of 5-azacytidine for 24 hours. Phenotypically, the cells are organized in bundles which fuse to form multinucleated cells. These cells express sarcomeric tropomyosin and desmin, both of which are not expressed in the undifferentiated cells.
The development profile of AFS cells differentiating into myogenic lineages mirrors a characteristic pattern of gene expression reflecting that is seen with embryonic muscle development\textsuperscript{63, 64}. With this protocol, $\textit{Myf6}$ is expressed at day 8 and suppressed at day 16. $\textit{MyoD}$ expression is detectable at 8 days and suppressed at 16 days. Desmin expression is induced at 8 days and increases by 16 days\textsuperscript{65, 66}.

\textit{Neuronal}

The AFS cells were induced in DMSO, butylated hydroxyanisole (BHA), and neuronal growth factor\textsuperscript{67, 68}. After 2 days the cells were returned to AFS growth medium lacking DMSO and BHA but still containing NGF. The cells cultured in neurogenic conditions change their morphology within the first 24 hours. Two different cell populations are apparent: morphologically large flat cells and small bipolar cells. The bipolar cell cytoplasm retracts towards the nucleus, forming contracted multipolar structures. Over the subsequent hours, the cells display primary and secondary branches and cone-like terminal expansions. The induced cells show a characteristic sequence of expression of neural-specific proteins. At an early stage the intermediate filament protein, nestin, which is specifically expressed in neuroepithelial stem cells, is highly expressed. The expressions of $\beta$III-tubulin and glial fibrillary acidic protein (GFAP), markers of neuron and glial differentiation, respectively, increases over time and seems to reach a plateau at about 6 days\textsuperscript{69}. The cells cultured under neurogenic conditions show the presence of the neurotransmitter glutamic acid in the collected medium. Glutamic acid is usually secreted in culture by fully differentiated neurons\textsuperscript{70}.

To induce the differentiation of AFS cells into dopaminergic cells a two-stage induction procedure has been designed. AFS cells are first seeded on fibronectin coated plates and incubated in DMEM/F12 medium supplemented with N2 and bFGF for 8 days. Under these conditions over 80\% of cells showed expression of nestin, a marker of neural stem cells. The cells then were transferred to conditions biasing to production of dopaminergic neurons \textsuperscript{71}. Under these conditions, a fraction of these cells assumed a characteristic pyramidal morphology. Gene expression analysis determined the expression of GIRK2 gene, a marker of dopaminergic neurons. After induction with another neurogenic differentiation protocol using NGF, AFS cells acquired the ability to secrete the excitatory neurotransmitter L-glutamate in response to stimulation by potassium ions.
5.1.3 IMPLANTATION AFS CELLS IN VIVO AND IN ANIMAL MODELS OF REGENERATION

To investigate if differentiated hAFS cells can truly contribute to the regenerative process of a damaged organ or tissue or to new tissue growth, several animal models were used.

Although before any in vivo experiment or therapeutical application, non-tumorigenic behavior in vivo needs to be confirmed. To investigate this, immunodeficient mice were used and cells implanted in an immunoprivileged site, the testis, in order to avoid any residual immune surveillance that these mice could have.

Certain cells differentiated in culture from human AFS cells are capable of integrating into normal appearing tissue structures when transplanted in vivo, under conditions promoting regeneration or new growth. Endothelial differentiated hAFS cells were challenged to participate in the neovascularization of a tumour. Myoblast differentiated hAFS were investigated in a skeletal muscle injury model induced by cardiotoxin. Similarly, hepatocyte differentiated hAFS were challenged to contribute to liver regeneration after partial hepatectomy.
5.2 MATERIALS AND METHODS

5.2.1 Teratoma analysis
Cells of one cloned human AFS cell line (hAFS-A1 at passage 14) labelled with MMP-nlacZ, pseudotyped with VSV-G viral vector (Harvard Gene Therapy Initiative, Harvard Medical School, Boston, MA) and mouse embryonic stem cells (mES 129 at passage 25) were injected into the scrotal pouch of 8-week-old male rag2−/− C57BL/6 mice (Taconic Inc., Hudson, NY, USA). All animal care and experimental procedures were done in full compliance with the Wake Forest University Institutional Animal Care and Use Committee, as well as all State and Federal Guidelines. Each mouse was injected with 5x10^6 cells of each cell line using 0.9% sodium chloride solution (Abbott Laboratories, North Chicago, IL, USA) as vehicle. One to three months after injection, dependent on tumour appearance, the mice were sacrificed and the scrotum removed. Testis and tumours (where present) were fixed for 2h at 4°C with 2% paraformaldehyde (Polysciences Inc., Warrington, PA, USA), 0.2% glutaraldehyde, 0.01% sodium deoxycholate and 0.02% NP-40 (Sigma-Aldrich, St. Louis, MO, USA) in PBS 1x (Gibco-Invitrogen, Carlsbad, CA, USA). After fixation, tissues were developed for 24 h at 30°C in solution with 0.1% X-Gal (Roche Applied Sciences, Indianapolis, IN, USA) 2mM magnesium chloride, 0.02% NP-40, 0.01% sodium deoxycholate, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide (Sigma-Aldrich, St. Louis, MO, USA). After X-Gal solution development, tissue was processed in a Shandon Citadel 1000 Tissue Processor (Thermo Electron Corporation, Waltham, MA, USA) and paraffin embedded for sectioning. Histological examination was done using hematoxylin and eosin staining.

5.2.2 Myoblast Implantation
Cloned hAFS-A1 cells were cultured in induction medium promoting myogenic differentiation (as described previously) and 5x10^5 cells previously labeled with Ad6-nlacZ vector, encoding β-galactosidase (β-Gal) modified with a nuclear localization signal (Harvard Human Gene Therapy Initiative, Harvard Medical School, Boston, MA) were injected using Matrigel HC (BD Biosciences, San Jose, CA, USA) as a vehicle in male nu/nu (nude) mice (Charles River Laboratories Inc, Wilmington, MA) 8 weeks old and 24h after injection with 30 µl of Cardiotoxin I solution (Sigma-Aldrich, St. Louis, MO, USA) at 10^-5 M in deionized water on both hindlimbs posterior tibialis skeletal muscle. Right hindlimb muscle was used as control.
Before cell injection, both hindlimbs were γ-irradiated with 25 gray by a JL Shepherd Mark II Cesium-137 Irradiator. 100 µl of 0.9 % sodium chloride solution (Abbott Laboratories, North Chicago, IL, USA) were injected in the right hindlimb muscle as control. Samples were collected for histological analysis after 1 (n=3) and 3 weeks (n=3). The dissected posterior tibialis skeletal muscles (hAFS injected and control) were developed in X-Gal solution (as described above). After development, tissue was processed and sectioned. Sections were stained with hematoxylin and eosin and immunostained with mouse anti-sarcomeric tropomyosin (Sigma-Aldrich, St. Louis, MO, USA).

5.2.3 Endothelial Cell Implantation
Cloned hAFS-A1 cells were cultured in induction medium promoting endothelial differentiation, and then 5x10⁵ cells were injected together with 2x10⁶ cells of human pancreatic carcinoma cells Hs-776T (HS-VF), engineered to over-express VEGF, into immune deficient nu/nu (nude) mice (Charles River Laboratories Inc, Wilmington, MA). The human cells were marked with the nlacZ gene with a nuclear localization β-galactosidase, introduced via a defective adenoviral vector (Harvard Human Gene Therapy Initiative, Harvard Medical School, Boston, MA), to allow their detection using a chromogenic β-galactosidase (β-Gal) substrate (X-gal). After 1 and 4 weeks tumours were collected for histological analysis. All samples were developed with X-Gal solution, before tissue processing and sectioning as described above. Sections were stained with hematoxylin and eosin. To detect mouse blood vessels, sections were immunostained with rat monoclonal anti-mouse CD31 antibody (BD Pharmingen, Franklin Lakes, NJ, USA).

5.2.4 Hepatocyte Implantation
Cloned hAFS-A1 cells were cultured in induction medium promoting hepatic differentiation (as described previously). Immune deficient nu/nu (nude) mice (Charles River Laboratories Inc, Wilmington, MA) were submitted to a partial hepatectomy (70 %) before injection of 5x10⁵ cells previously marked with the nlacZ gene with a nuclear localization β-galactosidase, introduced via a defective adenoviral vector (Harvard Human Gene Therapy Initiative, Harvard Medical School, Boston, MA). These cells were injected in the parenchyma of the remaining liver lobe.
(right lobe) using 0.9 % sodium chloride solution (Abbott Laboratories, North Chicago, IL, USA) as vehicle.

Samples were collected for histological analysis after 1 and 3 weeks. All samples were developed with X-Gal solution, before tissue processing and sectioning as described above. Sections were stained with hematoxylin and eosin and immunostained with mouse monoclonal anti-albumin antibody (Sigma-Aldrich, St. Louis, MO, USA).
5.3 RESULTS

Tumor formation was observed in the scrotal pouch of mice implanted with mES 129 cells as early as 4 weeks. As expected, all mice injected with mES 129 generated massive tumors. After histological examination, several differentiated structures/tissues were observed within the tumors (Figure 5.1A and 5.1B), undeniably confirming their embryonic origin and their classification as teratomas. The mice injected with hAFS, didn’t develop any tumors within the 3 month range of the study. No β-galactosidase positive cells could be observed in the histological sections of the testis and surrounding tissues (Figure 5.1C). Nonetheless, some scattered small vesicular X-Gal (blue) staining could be observed in some areas of the epididymis (Figure 5.1D). This blue staining was not nuclear localized, what indicates some sort of staining artifact. This experiment confirms the non-tumorigenic nature of hAFS cells that even in immunodeficient mice in immunoprivileged sites don’t generate tumors.

Figure 5.1 | Histological analysis of teratomas and testis of mice injected with mES and hAFS. Teratomas were found in mice injected with mES cells and were analyzed by histological examination and stained with hematoxylin and eosin, showing a stratified epithelium tubular
structure (A) and a cell mass of chondroid tissue (B). Mice injected with hAFS didn’t form any tumors and histological analysis of testis, revealed normal tissue architecture without stem cell incorporation (C). In some areas of the epididymis, non specific scattered X-Gal staining could be observed, probably due to a staining artifact.

When hAFS are differentiated into myoblasts, β-Gal positive myofibers could be clearly observed in histological sections of the posterior *tibialis* skeletal muscle damaged with cardiotoxin I (Figure 5.2A) one week after cell implantation. Immunohistochemical staining with antibody anti-sarcomeric tropomyosin confirmed the skeletal muscle identity of these labeled myofibers (Figure 5.2B). The integration of hAFS in the damaged skeletal muscle of this animal model shows that differentiated hAFS can identify the microenvironmental cues produced in regenerating muscle and contribute to the repair of the injured tissue. The exact mechanism/biology used by these cells in this regenerative process remains to be elucidated.

**Figure 5.2** | Histological analysis of injured skeletal muscle and VEGF secreting tumor cells injected with hAFS cells differentiated into myoblasts and endothelial cells, respectively. After cardiotoxin I injection, the hAFS cells injected into mice skeletal muscle incorporated in the
damaged myofibers area. β-gal positive myocytes can be clearly observed in a hematoxylin and eosin staining (A). These blue myofibers express also sarcomeric tropomyosin, confirming their skeletal muscle identity (B). In the retrieved tumors, it could be observed some β-gal positive cells (C). After CD31 staining to identify vascular structures, it could be observed blue staining cells aligned with the CD31 expressing cells, indication of possible contribution of hAFS cells into these newly formed vascular structures (D).

The implanted pancreatic cancer cells developed a detectable tumour as early as 2 weeks after injection and up to 4 weeks. Blood vessels growing in the tumour were visualized by immunohistochemical staining with an antibody to mouse CD31. Staining for β-Gal (blue) shows that human endothelial cells derived from hAFS cells, co-localize with these vascular structures. This provides some evidence that hAFS cells have the potential to integrate into these new blood vessels (Figure 5.2D) and contribute to tumour angiogenesis.

One week after hAFS cell injection into a regenerating liver, several extensive clusters of β-Gal positive cells could be observed throughout the whole liver (Figure 5.3A and 5.3B). Immunohistochemical staining for albumin showed intense expression within one of the clusters of β-Gal positive cells (data not shown). Although cell nuclei imaging with PI was quite difficult in these cell clusters, due to the intensity and darkness of the blue staining. Nevertheless, this experiment showed that hAFS differentiated into hepatic lineage engrafted in regenerating hepatic tissue after partial hepatectomy. However, due to the substantially different mechanisms of hepatic tissue repair, other injury models should be used to clarify and expand the potential of hAFS in liver disease/regeneration.
Figure 5.3 | Histological analysis of regenerating mouse liver after hAFS cell injection. One week after partial hepatectomy and hAFS cell injection, extensive clusters of β-Gal positive cells could be observed in eosin (A) and hematoxylin and eosin stainings (B).
5.4 DISCUSSION

The use of embryonic stem cells has been an intense debate with ethical controversies. Stem cells in amniotic fluid may represent an attractive alternative to embryonic and adult stem cells because of their apparent advantages of accessibility and multipotentiality over embryonic and adult stem cells, respectively.

There are many clinical situations that one could predict amniotic fluid stem cells be used for. For example, that one could foresee amniotic fluid stem cells being used to repair congenital anomalies. The cells could be used for surgical reconstruction of birth defects in the neonatal period\textsuperscript{76}. A small amount of amniotic fluid may be sufficient to yield enough cells to prepare and engineered tissue construct for implantation ready upon birth\textsuperscript{77, 78}. An example is a recent study showing experimental diaphragmatic hernia repair with autologous tendon engineered from mesenchymal amniocytes\textsuperscript{79}.

The \textit{in vitro} and \textit{in vivo} experiments completed so far provide solid evidence of the safety and regenerative capability of these stem cells\textsuperscript{80}, although further \textit{in vivo} studies need to be performed to expand the potential of hAFS cells in human medicine. In the particular case of the liver, combination of hAFS derived endothelial and hepatic cells constitute a new alternative source for liver cell therapies and liver tissue engineering. This is further emphasized by the fact that the most important factor affecting clinical hepatocyte transplantation is a lack of donor availability\textsuperscript{81}. Also, clinical evidence shows that adult hepatocyte transplantation benefit is transient in its therapeutic effects, due to the limited capacity of hepatocyte proliferation, reduced cell engraftment after transplantation and immune surveillance\textsuperscript{82}. Hepatocyte progenitor cells constitute potentially a better approach for transplantation. Considering the properties of hAFS, differentiation of these cells in hepatic progenitor cells is of capital importance, potentially increasing cell availability and immune compatibility. We expect that in the near future, amniotic fluid stem cells will become a relevant, vital tool in stem cell research, tissue engineering, and regenerative medicine.
5.5 CONCLUSION

AFS cells isolated from amniotic fluid are an exciting contribution to the field of stem cell biology and regenerative medicine. The discovery of these cells has been recent, and a considerable amount of work remains to be done on the characterization and use of these cells in vivo. In the future, banking of these stem cells could provide a convenient source for autologous therapy by matching of histocompatible donor cells with recipients. AFS cells could represent an attractive and abundant, noncontroversial source of stem cells for regenerative medicine.
5.6 BIBLIOGRAPHY


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