STEM CELLS

REGENERATIVE MEDICINE

Concise Review: Amniotic Fluid Stem Cells: The Known, the Unknown, and Potential Regenerative Medicine Applications

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Key Words. Amniotic fluid stem cell • Fetal stem cell • Cell therapy • Cell transplantation • Tissue engineering • Regenerative medicine

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Received January 5, 2016; accepted for publication October 1, 2016; first published online in STEM CELLS *EXPRESS* December 23, 2016.

1066-5099/2016/$30.00/0

http://dx.doi.org/10.1002/stem.2553

Available online without subscription through the open access option

ABSTRACT

The amniotic fluid has been identified as an untapped source of cells with broad potential, which possess immunomodulatory properties and do not have the ethical and legal limitations of embryonic stem cells. CD117(c-Kit)+ cells selected from amniotic fluid have been shown to differentiate into cell lineages representing all three embryonic germ layers without generating tumors, making them ideal candidates for regenerative medicine applications. Moreover, their ability to engraft in injured organs and modulate immune and repair responses of host tissues, suggest that transplantation of such cells may be useful for the treatment of various degenerative and inflammatory diseases. Although significant questions remain regarding the origin, heterogeneous phenotype, and expansion potential of amniotic fluid stem cells, evidence to date supports their potential role as a valuable stem cell source for the field of regenerative medicine. STEM CELLS *2017;35:1663–1673*

SIGNIFICANCE STATEMENT

The amniotic fluid is an under-utilized source of stem cells, with therapeutic potential in the field of regenerative medicine. Stem cells from the amniotic fluid can be isolated and expanded easily, and have the ability to differentiate into a various cell types without the risk of tumorigenesis. Emerging evidence from experimental models of disease has generated great interest in potential clinical applications of amniotic fluid stem cells for human tissue repair and regeneration. The latter, in combination with the lack of ethical concerns associated with other stem cell sources, makes stem cells derived from the amniotic fluid prime candidates for the development of novel therapies against a wide range of congenital and acquired human disorders.

INTRODUCTION

Recent discoveries in regenerative medicine have intensified the search for new stem cell sources with therapeutic potential. In recent years, the amniotic fluid has been recognized as alternative source of stem cells for tissue regeneration. Adult stem cells are limited in their potential and, even after reprogramming may maintain epigenetic modifications, which may limit their application. Fetal stem cells may overcome these limitations and, while at birth the umbilical cord and placenta are significant alternatives, the amniotic fluid is an appealing cellular reservoir during gestation. In addition to the potential clinical utility of the amniotic fluid, ethical concerns associated with its isolation are minimal [1], as it can be collected safely during second trimester routine amniocentesis (at 14-16 weeks gestation), third trimester amnioreduction (at 28 weeks or later), or caesarean section (end of gestation).

The amniotic fluid serves as a protective liquid for the developing fetus, and provides mechanical support as well as required nutrients during embryogenesis [2]. It is composed mainly of water, chemical substances, and cells [3]. These cells are heterogeneous in morphology, in vitro and in vivo characteristics [4]. They are mostly of fetal origin (skin, respiratory, intestinal, and urinary tracts), as well as the amniotic membranes and connective tissues. Cells in the amniotic fluid increase in number with gestational age, with the exception of pathological conditions affecting cell turn-over (e.g., low counts in cases of urogenital atresia, and increased counts in spina bifida) [5]. In addition to cell count variability, the amniotic fluid contains different cellular subpopulations (epithelioid, "amniotic" and fibroblastic type), which vary in proportion according

to gestational age. Amniotic fluid mesenchymal stem cells (AFMSC) are particularly interesting because of their potential therapeutic applications, and several methods of isolation and expansion have been described. These are mostly based on plastic adherence of unselected populations of amniotic fluid cells in serum-rich conditions without feeder layers from small volumes (2-5 ml) of second and third trimester amniotic fluid [6–8]. AFMSC exhibit a broad differentiation potential toward mesenchymal lineages, and have the ability to differentiate toward adipogenic, osteogenic, and chondrogenic lineages [8–10] Moreover, AFMSC can be a suitable cell source for tissue engineering and their ability for the repair of muscle, cartilage and bone defects have been tested in established animal models [12–15]

Additionally to AFMSC, CD117(c-Kit)+/Lin− amniotic fluid stem cells (AFSC) have been described [16]. AFSC are broadly multipotent as they are able to differentiate not only into mesoderm-derived (bone, fat, cartilage, muscle, hematopoietic), but also in nonmesodermal lineages (endothelial, hepatic, neuronal) [7]. In addition, AFSC are nontumorigenic, and do not form teratomas in vivo when injected in immunodeficient mice [17]. Together these characteristics highlight the cellular plasticity of AFSC, and support a broadly multipotent (vs. pluripotent) phenotype. We aim to discuss here the advantages, applications and limitations of AFSC. The lack of tumorigenicity in an easily obtainable primitive stem cell type, and the ability to generate progenitors of a wide range of lineages render AFSC attractive candidates for regenerative medicine-based treatments against both congenital and acquired disorders [1–5, 16].

The Known

The first suggestion that stem cells may be present in the amniotic fluid was based on the observation that amniotic fluid-derived cells expressed the skeletal muscle protein dystrophin when exposed to the supernatant of rhabdomyosarcoma cell lines while in culture [18]. Subsequently, amniotic fluid-derived cells were shown to have a mesenchymal stem cell surface marker profile, as well as be able to differentiate to osteocytes, adipocytes and fibroblasts [9]. Prusa et al. were the first to demonstrate that Octamer Transcription Factor-4 (Oct-4) is expressed in amniotic fluid-derived cells at both transcriptional and protein levels [19]. Remarkably, Karlmark et al. showed that some amniotic fluid cells were able to activate the Oct-4 and Rex-1 promoters, providing more evidence on the presence of stem cells in the amniotic fluid [20]. Subsequently, we and others, used CD117 (c-Kit; type III tyrosine kinase receptor for stem cell factor with essential roles in gametogenesis, melanogenesis, and hematopoiesis) in order to isolate the undifferentiated population from the amniotic fluid [17, 21]. These CD117-expressing cells are a heterogeneous population, have been isolated from small [7, 21–23] and large animals [24] as well as humans [17, 25], and are known as AFSC.

Isolation

AFSC can be isolated from amniotic fluid samples collected at any gestational age. However, AFSC isolated from first trimester amniotic fluid are thought to be more primitive (share 82% transcriptome identity with embryonic stem cells (ESC), more likely to be of germ origin), but they are not accessible in the autologous setting, while clinically accessible mid- or late-trimester AFSC may still maintain relevant therapeutic characteristics [26, 27]. Most of the studies to date have used similar isolation protocols [17], and have focused on mid- and late-trimester AFSC. In brief, freshly isolated amniocytes are plated on glass coverslips or Petri dishes, in media containing high concentration of serum (10%-15% fetal bovine serum/FBS; Chang C-, Amniomax-, and Dulbecco's modified Eagle's medium/DMEM-based media have been described) [28]. After 7-10 days, a small minority of cells attach, forming compact colonies; approximately 1% of these cells are CD117+ and can be isolated via magnetic or fluorescent cell selection/sorting (MACS or FACS) with high purity (>90%) [17].

In addition to this "attachment to plastic/CD117 selection" protocol, AFSC can be isolated directly from amniotic fluid samples based on expression of CD117 ("direct CD117 selection" protocol) [21]. This may be difficult after amniocentesis because of the limited amount of amniotic fluid that can be collected, but it may the best method in case of amniodrainage or for amniotic fluid collected at delivery (large amounts of fluid collected may make the attachment to plastic/CD117 selection protocol impractical. To our experience, high purity CD117+ human AFSC isolation in this setting can only be achieved using FACS sorting, as the large amount of cellular debris and small number of CD117+ cells do not allow efficient enrichment by MACS (this is less of an issue in mice). Using this approach, we and others have shown that the absolute number of AFSC as well as the proportion they represent out of the total live cells in the amniotic fluid show significant variability [21]. The absolute number of CD117+ AFSC increases with gestation in humans [21], but remains relatively stable in the mouse [21]; in contrast, the proportion of CD117+ AFSC (out of total live cells in amniotic fluid) peaks in mid-gestation in human and mouse (approximately 1%-8% of live cells in second trimester amniotic fluid), but is reduced thereafter (approximately 0.5%-4% of live cells in third trimester amniotic fluid) [21, 26]. From a typical second trimester human amniocentesis sample (2-3 ml), we can obtain 0.5-3 $\times$ 10^3 live, CD117+ AFSC by FACS sorting. In mice, we can obtain approximately 1 $\times$ 10^4 live CD117+ per fetus at a gestational age of 13-14 days (E13-E14; term E21) [1].

An increasing number of publications describe AFSC isolation using culture conditions alone (no CD117 selection at any stage). In the majority of these studies, amniocytes are plated in tissue culture-treated dishes in high-serum media (DMEM, with 10%-20% FBS, with or without basic fibroblast growth factor/FGF-2) [29], and cells have largely mesodermal potential (i.e., they can generate adipogenic, chondrogenic, osteogenic, and myogenic lineages; likely to be AFMSC) [11, 30], although some authors have reported multi-lineage differentiation potential not restricted to mesoderm (including neural differentiation, as well as embryoid body formation) [31–35]. In other cases, it has been observed that cells with similar potentials to AFSC can be derived using precise culture methodology but without positive selection [25, 27]. AFSC, derived by positive selection or by expansion conditions, have a cellular phenotype that is between ESCs and adult mesenchymal stem cells (MSC; see Cellular Characteristics and Fig. 1 below) [17]; this is in contrast to other amniotic fluid-derived cells with a purely mesenchymal phenotype [29], with obvious

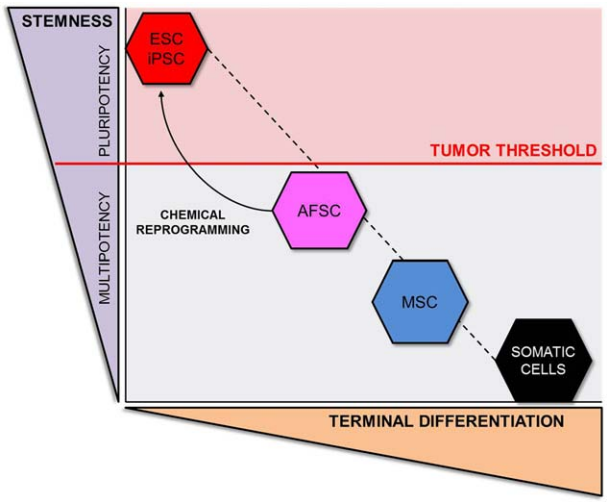

Figure 1. AFSC have an intermediate cellular phenotype between ESCs/iPSC and MSC. This is supported by expression of both markers/transcription factors of pluripotency and mesenchymal commitment, as well as their broadly multipotent nature (ability to differentiate to lineages representing all three embryonic germ layers, but do not form tumors). AFSC (in contrast to MSC) can be reprogrammed to pluripotency using a chemical (gene-free) approach. The *x*-axis in the figure represents increasing terminal differentiation, and the *y*-axis represents increasing "stemness." Abbreviations: AFSC, amniotic fluid stem cells; ESC embryonic stem cells; iPSC induced pluripotent stem cells; MSC, mesenchymal stem cells.

implications regarding potential regenerative medicine applications. For the rest of the present review, we will focus our discussion on CD117+ AFSC only.

Cellular Characteristics

The "intermediate" cellular phenotype of AFSC (between ESC and adult MSC; Fig. 1) is supported by expression of both markers/transcription factors of pluripotency and mesenchymal commitment. Early pregnancy (first trimester) human AFSC are thought to have a more primitive phenotype as evident from the 82% homology in transcriptome identity with ESC [26]. They are a heterogeneous population, with a bimodal distribution of cell size (from 8 μm to 15 μm; mean 12.0 ± 0.3 μm). The smaller cells grow in colonies, express Oct-4, c-Myc, Sox2, and Nanog, and are stage specific embryonic antigen 3 positive (SSEA3+), SSEA4+, Tra-1-60+, Tra-1-81+, and alkaline phosphatase+ (ALP+) [25]. The larger cells are SSEA3- with a fibroblastic morphology, and do not express pluripotency/ESC transcription factors and cell membrane markers; they are thought to have an accessory role supporting the growth of SSEA3+ AFSC (similar to what is observed in human ESC) but their precise function remains to be determined [25]. The fetal origin of first trimester AFSC has been confirmed in samples from male fetuses by the presence of the SRY gene [25]. Interestingly, CD117+, SSEA3+ AFSC have been shown to share common characteristics with primordial germ cells (PGC) or PGC precursors, expressing c-Kit, FGF-8, Sox17, STELLA, DAZL, NANOS, VASA, SSEA1, FRAGILIS, and PUM2; these observations provide some supportive evidence for a PGC origin of human AFSC [24]. In addition to pluripotency markers, first trimester AFSC express high levels of several MSC markers including CD29, CD44, CD73, CD90, CD105. Human Leucocyte Antigen (HLA) is low or negative (both class I and II) [25]. They are able to form embryoid bodies (and hence all three germ linages) in vitro, but do not form teratomas when injected in immunocompromised mice, consistent with a broadly multipotent phenotype [25].

Second and third trimester human AFSC (isolated with the attachment to plastic/CD117 selection protocol) express c-Myc and Oct-4 but do not express Nanog and Klf4. They are also SSEA4+, but SSEA3, Tra-1-60, Tra-1-81, and ALP are absent [27]. Their mesenchymal phenotype is evident by high levels of expression of MSC markers on the cell membrane including CD29, CD44, CD73, CD90, CD105, as well as CxCR4, stromal cell-derived factor 1 receptor, CD146, CD166 and CD184. HLA class I (HLA-ABC) is expressed (expression is low), but the cells are negative for HLA class II (HLA-DR). Second trimester AFSC can differentiate into mesoderm-derived lineages (bone, cartilage, muscle, fat) with ease, but also in endodermal [26] (e.g., hepatic; albumin, α-fetoprotein, and c-met receptor expression following growth in hepatocytic medium) and ectodermal (e.g., neuronal; neural stem cells identified by nestin expression, and dopaminergic neurons identified by G-protein-gated inwardly rectifying potassium channel-2 gene expression following growth in neurogenic medium) lineages, although the latter have proven to be challenging [17]. A recent study from our group showed that second trimester AFSC cannot form embryoid bodies, unless exposed to ESC culture conditions (see Culture section), and do not form teratomas when transplanted in immunocompromised mice [17]. These observations suggest that in comparison to first trimester human AFSC, second (and third) trimester AFSC (at least when isolated with the attachment to plastic/CD117 selection protocol) are more mesodermally-committed but retain some "ESC-like" plasticity and broadly multipotent characteristics. Similar to first trimester AFSC, second trimester AFSC are of fetal origin (confirmed by SRY gene analysis) and express migratory PGC markers, albeit at significantly lower levels; this is consistent with a PGC origin of AFSC as previously hypothesized [27].

Up to now, we have intentionally avoided any reference to expression of hematopoietic markers [CD45 (mouse and human), CD34 (mainly human but also mouse), CD14 (human), Sca-1 (mouse)] in AFSC of any gestational age. We have done this, as we feel it is important to highlight a (to our view) significant detail that may create some confusion in the field. A seminal study by Ditadi et al. [20], as well as subsequent work in our laboratory demonstrated that CD117+ and hematopoietic lineage- (Lin-) human and mouse mid trimester AFSC isolated by the direct CD117 selection methodology (FACS/MACS sorting) express hematopoietic markers (CD45+/high, CD34+/low, Sca-1+/low) as well as key hematopoietic regulators (e.g. GATA1, GATA2, Lmo2) at levels that are comparable to fetal hematopoietic stem cells (HSC; evidence available in mouse only) [21]. More importantly, murine and human AFSC isolated by this method have been shown to have significant multi-lineage hematopoietic potential in vitro and in vivo (see Potential Regenerative Medicine Applications section). In addition to markers of hematopoiesis, murine mid-trimester (E13-E14) CD117+ AFSC express Oct-4, c-Myc (but only low levels of Sox2, and no expression of Nanog) as well as the mesenchymal markers CD44, CD90, and CD105. It is not clear at present whether these freshly isolated AFSC with hematopoietic potential represent a distinct

stem cell group (of fetal hematopoietic origin) within the heterogeneous CD117+ AFSC population, or if all broadly multipotent AFSC express hematopoietic markers at some stage (prior to selection by adherence). What is clear though, is that human AFSC of any gestational age isolated by the attachment to plastic/CD117 selection protocol do not express hematopoietic markers [17, 25, 27, 36], which could be due to "selection by attachment/culture" of the mesenchymal-type AFSC (AFSC with hematopoietic characteristics either differentiate or die). Due to the significant clinical implications of identifying a novel source of fetal hematopoietic stem cells with potential for therapy, the determination of the hematopoietic characteristics of fresh, FACS/MACS-sorted, CD117+ AFSC (and more importantly how to maintain them in culture; see Culture section) is a major research focus in our laboratory (also see The Unknown section below).

Finally, murine and human AFSC of any gestational age and isolated by any methodology, do not express endothelial markers, including CD133 and CD31 [17, 25–27]. However, a recent study from our laboratory has shown that second and third trimester human AFSC (isolated by the attachment to plastic/CD117 selection protocol) express ETV2 and FLI1 (but not ERG1), which are members of the E-twenty six transformation family of transcription factors directing angiogenesis and endothelial fate [26].

Culture

There are currently two distinct culture protocols for AFSC. One involves culture in feeder layer-free, serum rich conditions ("mesenchymal-type" culture), and the other culture in ESC conditions ("embryonic-type" culture).

The mesenchymal-type culture methodology involves plating of CD117+ AFSC on Petri dishes (or nontissue culture treated flasks) at a density of around 3-5 $\times$ 10^3 cells per cm^2.The culture medium consists of Chang medium B and C, α-minimum essential medium and FBS (15%) (DMEM with 10% FBS has also been used) [17]. The medium is refreshed every 48 hours, and confluence is maintained at around 60% [17]. Culture of human AFSC of second or third trimester under these conditions allows significant expansion as a monolayer of cells with large spindle-shaped cytoplasm (clonal line doubling time of approximately 36 and 42 hours, respectively), as well as preservation of the broadly multipotent nature of these cells. Rodent AFSC closely resemble human AFSC in their growth properties and capacity for in vitro differentiation. Although expression of CD117 declines (and ultimately disappears) with this culture methodology [26], expression of some of the pluripotency (c-Myc, Oct-4, SSEA4) [36, 37], endothelial (ETV2, FLI1), and all of the mesenchymal (CD29, CD44, CD73, CD90, CD105) markers is maintained [26]. Growth kinetics analysis of cultured AFSC has shown exponential growth, reaching up to 250 population doublings without any signs of slower proliferation or senescence [17, 25, 27]. This high expansion potential has been associated with long telomeres and active telomerase [25, 27], and whole genome array analysis has not identified any aberrations larger than 100 kb, (besides known benign copy number variations) indicating karyotypic normality and stability after long-term in vitro cell expansion [17, 25]. AFSC cultured this way do not form embryoid bodies or teratomas, but can differentiate into mesodermal and nonmesodermal lineages under appropriate differentiation conditions, although functional in vivo lineage differentiation toward endoderm and ectoderm has not yet been fully demonstrated [17, 26]. Interestingly, hypoxia (culture in 5% instead of 21% O_2) has recently been shown to enhance both the proliferation kinetics as well as the endothelial commitment of second and third trimester AFSC, with the effects being more marked in late-gestation AFSC [26].

The embryonic-type culture involves expansion of human AFSC using Stemedia NutriStem XF/FF and Matrigel-coated plates (similar to what is used for human ESC cultures). This culture methodology has been attempted with first and second trimester AFSC, and plating densities of 5-10 $\times$ 10^3 cells per cm^2 have been used [25, 27]. Medium change is performed every 24 hours and confluence is maintained to under 70% [25, 27]. Using these conditions, it has been possible to expand first trimester human AFSC in colonies of packed cells (doubling time of approximately 17 hours) while maintaining expression of both pluripotency (c-Myc, Oct-4, Sox2, Nanog, SSEA3, SSEA4, Tra-1-60, Tra-1-81), as well as mesenchymal markers (CD29, CD44, CD73, CD90, CD105) [16]. These cells form embryoid bodies, but not teratomas. Second trimester human AFSC can also be expanded in embryonic-type cultures, with concomitant upregulation of pluripotency markers (including NANOG, Klf4, SSEA3, Tra-1-60, and Tra-1-81) that are normally not expressed in freshly isolated AFSC or AFSC expanded under mesenchymal conditions [27]. Such cells seem to proliferate faster than mesenchymal-type AFSC (clonal line doubling time of 23 hours vs. 36 hours), but still maintain a normal karyotype and stable telomeres [25, 27]. Moreover, they have the ability to form embryoid bodies (but not teratomas), which is in contrast to what has been observed for AFSC in mesenchymal expansion conditions [25, 27]. More importantly, the effects of embryonic-type culture on second trimester AFSC are maintained even when cells are initially expanded in adherence (i.e., switching culture conditions leads to a switch in AFSC phenotype) [25, 27]. This cannot be attributed to detrimental effects of ESC-like conditions to a subset of fibroblastic cells (favoring the survival of more primitive cells), and further supports the phenotypic plasticity of AFSC [25, 27].

Our group has recently shown that murine CD117+/Lin− AFSC can be cultured in embryonic-type media on feeder layers of mitotically inactivated embryonic fibroblasts producing leukemia inhibitory factor [38]. AFSC are plated in high densities (10^5 per cm^2) and have a doubling time of approximately 40 hours. In addition to maintaining expression of pluripotency (c-Myc, Oct-4, Sox2, Klf4) and mesenchymal markers (CD44, CD73, CD90, CD105), this culture methodology also allows preservation of CD117 and hematopoietic marker expression (CD45, CD34, Sca-1; also GATA1, GATA2, Lmo2, and other key regulators of hematopoiesis; note that these cells were originally isolated by the direct CD117 selection protocol) [21]. In vivo differentiation of these cells has been demonstrated into mesodermal (muscle) [38] and hematopoietic lineages [21]. Whether the hematopoietic potential of human AFSC can also be maintained in culture remains to be determined (see The Unknown section below).

Finally, reprogramming to pluripotency AFSC is possible using ectopic expression of OKSM and other integrative and nonintegrative techniques [39, 40]. Fetal stem cells present some advantageous characteristics compared with their

neonatal and postnatal counterparts, with regards to cell size, growth kinetics, and differentiation potential, as well as in vivo tissue repair capacity. Moreover, first and second trimester CD117+ AFSC can also be converted into induced pluripotent stem cells (iPSC) in a transgene-free fashion by addition of the FDA approved chemical valproic acid (VPA) in the culture media [25]. VPA acts as an epigenetic modifier by remodeling chromatin through direct inhibition of histone deacetylase [25]. Chemical reprogramming (see Fig. 1) can be achieved relatively easily in first trimester AFSC (consistent with a more primitive phenotype), but requires prior exposure to embryonic-type culture conditions in order to be successful in second trimester AFSC (this is consistent with first trimester AFSC being more primitive than mid-gestation AFSC) [27]. Exposure of AFSC to VPA (1mM for 5 days) resulted in fully functional iPSC, that could form teratomas and were stable when VPA supplementation was withdrawn [25, 27].

The Unknown

The amniotic fluid has traditionally been considered a waste or, in the best scenario, a mechanical barrier to the fetus providing nutritional requirements for the development of the intestine [41] Subsequently (because of its accessibility), it has become the "gold standard" (from the 1970s onward, but probably not for much longer [42]) for acquiring fetal cells for genetic prenatal screening. For years, this has been the only clinical use of amniotic fluid-derived cells, and despite the discovery of a discreet AFSC population with potential for therapy, there are significant questions that have not been addressed to date and are summarized below.

What Is the Origin of AFSC?

Based on their morphological and growth characteristics, viable adherent cells from the amniotic fluid are classified into three main groups: epithelioid (33.7%), amniotic fluid (60.8%) and fibroblastic type (5.5%) [43]. In the event of fetal abnormalities other types of cells can be found in the amniotic fluid (e.g., neural cells in presence of neural tube defects and peritoneal cells in case of abdominal wall malformations). The majority of cells present in the amniotic fluid are terminally differentiated and have limited proliferative capabilities [5]. In the 1990s, however, two groups demonstrated the presence of small subsets of cells in the amniotic fluid harboring a proliferation and differentiation potential. First, Torricelli et al. reported the presence of hematopoietic progenitors in the amniotic fluid collected prior to week 12 of gestation [44]. Subsequently, Streubel et al. were able to differentiate amniotic fluid cells into myocytes, thus suggesting the presence of nonhematopoietic precursors in the amniotic fluid [18]. These results triggered new interest in the amniotic fluid as an alternative source of cells for therapeutic applications. While it has not been proven that pluripotent cells are actually present in the amniotic fluid, cells with various potentials have been successfully isolated [1, 11, 17]. Among those, CD117+/Lin− are broadly multipotent and have been shown to reliably differentiate beyond mesenchymal potential. Differently to what initially thought, they could originate from extra-embryonic tissue such as the placenta and then migrate to the AF. Moreover, because they express c-Kit, they may originate from fetal PGCs, neural crest, and/or hematopoietic stem cells, since all these cell types express this tyrosine kinase receptor during embryonic and fetal development [44–48]. There could also be differences among species because of differences in origin of the amniotic fluid and its composition [49]. For example, the persistence of yolk sac in mice could influence cellular composition of amniotic fluid and therefore results obtained using mouse AFSC need to be validated with human cells prior to clinical translation.

Is the Amniotic Fluid a Stem Cell Niche?

It has become quite clear that the amniotic fluid contains different types of stem cells and progenitors with a broad potential. It could be speculated that the amniotic fluid provides the signaling required for these cells to maintain their undifferentiated status, which would be consistent with a stem cell niche role for the amniotic fluid. When considering, for example, CD34+ cells it is quite striking that similar percentage (proportion of total cells) of these are present in the amniotic fluid surrounding the growing fetal lamb and the adult sheep bone marrow (BM) [50]. This could be due to the ability of the cells to grow in that particular environment. Interestingly, it could also be explained by the fact that the two potent mechanisms promoting cell differentiation, that is, cell-cell contact and cell-matrix contact do not influence cells floating in the amniotic fluid at very low concentration. Indeed, it has been reported that substituting serum with human amniotic fluid provides a growth milieu for adult bone marrow mouse HSC cultures in which differentiation and apoptosis are downregulated and multipotency is maintained [51]. More data is needed to understand if the amniotic fluid is capable of maintaining stemness (e.g., culture of AFSC in pure amniotic fluid or amniotic fluid-containing media), to determine if there is active stem cell homeostasis between the fluid and other fetal tissues and as well as the potential contribution of these cells to tissue repair/regeneration in the fetus.

Are AFSC Safe to Use in Patients?

Fetal stem cells retain capacity for proliferation and differentiation greater than that of their adult counterparts, however data reported so far do not describe spontaneous teratoma formation [7]. Indeed, Guillot's group have tested the potential of AFSC to form teratoma extensively but proved they could only achieve it when fully reprogrammed to iPSC prior to injection in immunocompromised mice [16]. This is now quite clear for both mouse and human cells, however there are no long-term experiments with cells, although these have been extensively expanded and differentiated to form functional tissues prior to implantation. To their advantage, they could actually be safer than postnatal cells because MSC in prolonged culture have been found to exhibit defects in genetic stability and differentiation capacity [52]. Epigenetic anomalies have been hypothesized to be a cause of these defects. Interestingly, previous studies have observed no irregularity in the epigenetic control system in early-passage AFSC, indicating that most likely the standard in vitro culturing of AFSC is safe for clinical application [53].

Can a Sufficient Amount of AFSC be Banked for Clinical Application?

Amniotic fluid can be collected in small volume from amniocentesis samples during the second trimester or in much

larger volume during amnio-drainage or caesarean section. Some cell types, such as epithelial cells, are more abundant, some others such as c-Kit+ AFSC are rarer (typically around 1% of live cells) and according to some, may be too much of a heterogeneous cell source, with high donor variations and therefore difficult to utilize for autologous cell therapy [54]. In order to obtain enough cells for therapy, AFSC would need to be expanded extensively. The favorable proliferation kinetics of AFSC, as well as the genetic stability and lack of senescence in these cells after long-term culture (see Culture section above) should allow the generation of clinically relevant AFSC numbers. In addition, reprogramming can be utilized to further improve both proliferative and differentiation capabilities. The latter is where AFSC could overcome limitations associated with adult derived iPSC. It is possible that fetal cells are more amenable to reprogramming and therefore safer because of their closer origin to embryonic tissue. This is in keeping with the demonstration that iPSC can be derived from AFSC without any genetic manipulation [25]. Regarding other cell type, HSC-type stem cells seem to be abundant (possibly one of the largest subpopulations within the heterogeneous AFSC population) in freshly harvested amniotic fluid [54]. Moreover, at least in mice, there is preliminary evidence that it is possible to expand AFSC in vitro while maintaining their hematopoietic characteristics [55]; however, limitations in their expansion potential remain similar to these observed in adult HSC. The latter represents a major obstacle to clinical application of AFSC in this setting.

Regenerative Medicine Applications

Human ESCs have been considered the gold standard for therapy due to their pluripotent characteristics, as well as the reproducible isolation and culture protocols available [56]. However, their clinical translation has been delayed by ethical concerns, as well as the significant risks of immunogenicity and tumorigenicity. The generation of iPSC allowed the stem cell community to overcome two key limitations of human ESC, namely ethical issues and immunogenicity [57]. Current limitations that have hindered utilisation of human iPSC in the clinic include the low efficiency of iPSC protocols and the risks of insertional mutagenesis/tumorigenesis associated with use of viral vectors [4]. The use of nonintegrating reprogramming/gene delivery methods (e.g., adenovirus or Sendai virus-based vectors, episomal plasmids) has addressed concerns regarding insertional mutagenesis, but the efficiency of reprogramming is generally lower compared to that achieved with lentiviral vectors [58]. Therefore, there is great potential to use AFSC for clinical translation, mainly due to their differentiation capabilities, in vitro culture characteristics, as well as the lack of tumorigenic potential and ethical concerns [4]. Moreover, if pluripotency is needed AFSC can be efficiently reprogrammed to generate iPSC in a transgene-free approach (see Culture section above), thus eliminating any risks of insertional mutagenesis [25]. Finally, as AFSC originate from the fetus, they could be used as an autologous stem cells source for prenatal and postnatal regenerative medicine applications. With this in mind, our group are actively developing biobanking and GMP-compliant culture protocols that may allow use of AFSC in the clinic. However, AFSC may also be useful to a larger number of patients where they could stimulate tissue repair and regeneration in an allogeneic setting. We discuss below how, beside direct differentiation, in various system their therapeutic effect could be related to paracrine [59] or immunosuppressive effects, with irradiated amniotic fluid cells capable in vitro to determine a significant inhibition of T-cell proliferation with a dose-dependent kinetics [60] Potential regenerative medicine applications of AFSC (as well as key supporting evidence from animal models) are summarized in Figure 2.

Cardiovascular System

Our group have investigated the cardiomyogenic potential of AFSC. We have shown that AFSC can express cardiomyocyte-specific proteins (e.g., atrial natriuretic peptide), as well as endothelial- (e.g., CD31) and smooth muscle cell-specific proteins (e.g., alpha-smooth muscle actin), when induced using appropriate culture conditions (e.g., coculture with cardiomyocytes) [4]. In subsequent in vivo experiments, intracardiac transplantation of rat AFSC (allogenic) in adult rats with cardiac ischemia-reperfusion injury (IR) improved left ventricular ejection fraction as assessed by magnetic resonance imaging (MRI) at follow-up three weeks post-IR, with evidence of a paracrine mechanism of action [22]. The paracrine therapeutic effect was confirmed in subsequent experiments in rat myocardial infarction (MI), in which intravascular transplantation of xenogenic/human AFSC and/or their conditioned media immediately post-MI (at the onset of reperfusion) resulted in cardioprotection [improved cell survival and decreased infarct size (from 54% to 40%)], with evidence supporting a role of AFSC-secreted thymosin beta-4 (Tβ-4), an actin monomer-binding protein with cardioprotective properties [61]. Tβ-4 has previously been implicated with cardioprotection in MI models that involved bone marrow-derived MSC (BM-MSC) injection [62].

We have also shown that AFSC may be beneficial in the setting of right heart failure secondary to pulmonary hypertension. When AFSC were injected intravascularly in rats, they homed to the heart and lung and reduced natriuretic peptide (BNP), a surrogate marker for heart failure, and pro-inflammatory cytokines. AFSC differentiated into endothelial and vascular smooth muscle cells forming micro-vessels, capillaries and small arteries, and there was a decrease in pulmonary arteriole thickness of about 35% [63]. Of relevance, in a seminal paper Rafii has demonstrated that is possible directly converting human mid-gestation amniotic fluid-derived cells into a stable and expandable population of vascular endothelial cells without utilizing pluripotency factors [64]. Notably, the expression of early endothelial transcription factors ckit+ AFSC prior differentiation. Moreover, in vivo, AFS cells from both second and third trimesters expanded in hypoxia were able to rescue the surface blood flow when locally injected in mice after chronic ischemia damage, and possessed the ability to fix carotid artery electric damage [26].

Gastrointestinal System

In recent experiments using a rat model of necrotizing enterocolitis (NEC), our group demonstrated that intraperitoneal administration of AFSC resulted in improved NEC clinical status at 4 days and survival at 7 days compared to controls (including BM-MSC, and PBS/vehicle), as well as significantly decreased peritoneal fluid accumulation (a surrogate marker

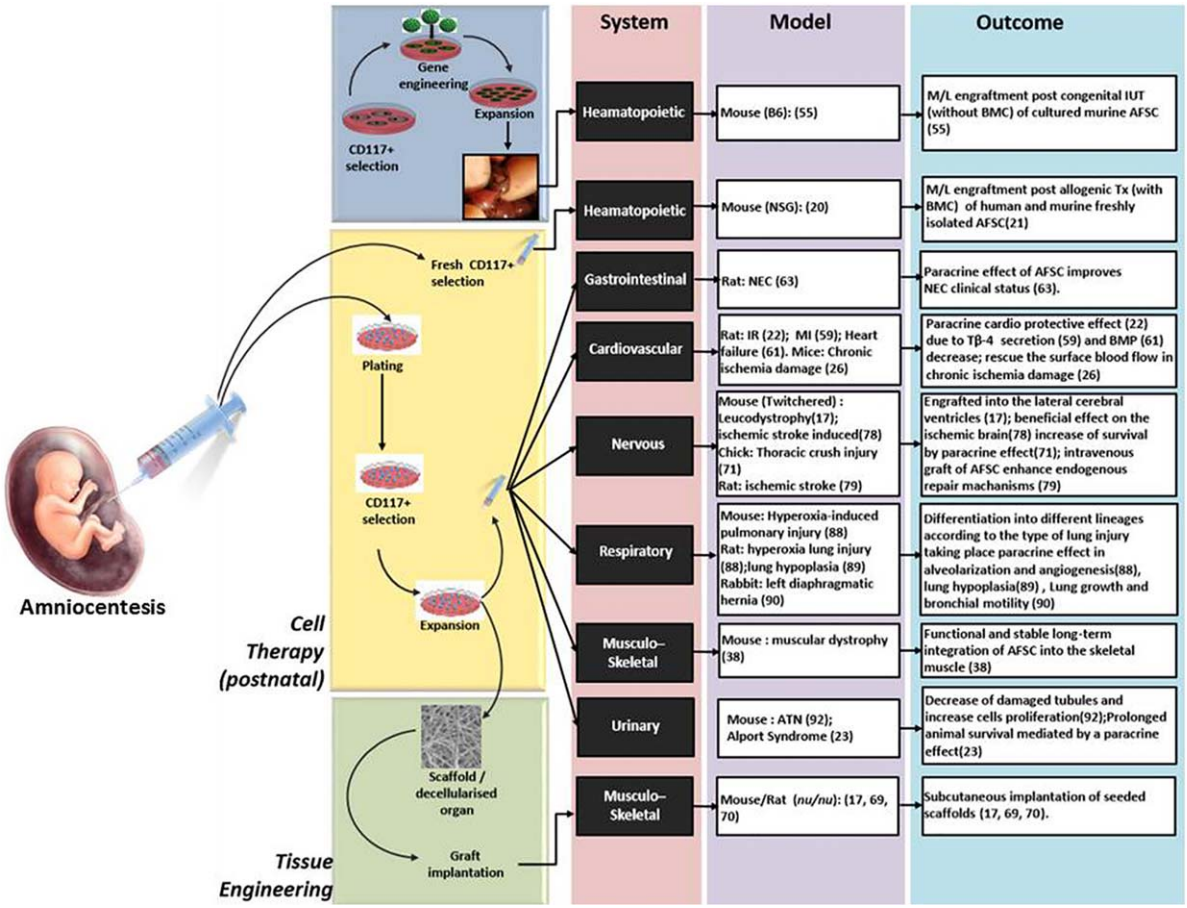

Figure 2. Summary of key supporting evidence from animal models for potential clinical applications of freshly isolated and expanded AFSC. Abbreviations: AFSC, amniotic fluid stem cells; ATN, acute tubular necrosis; BMC, bone marrow conditioning (irradiation); IR: ischemia-reperfusion; IUT, in utero transplantation; M/L, multi-lineage; B6, C57BL/6J ("black 6") mice; MI, myocardial infarction; NEC, necrotising enterocolitis; NSG, NOD-SCID/IL2rγ^{null} (immunocompromised) mice.

for NEC grade) on MRI [59]. Reassuringly, clinical results were corroborated by histological evidence, with improvements in villus sloughing/core separation/venous congestion in AFSC-treated animals. Elegant tracking experiments using GFP-expressing AFSC demonstrated preferential localisation of donor AFSC in intestinal villi; the low donor cell numbers alongside the great therapeutic effect of transplanted AFSC suggested a paracrine effect. The paracrine mechanism was confirmed and investigated in more detail in subsequent work that demonstrated the dependence of the salutary effects of AFSC transplantation in the setting of AFSC on cyclo-oxygenase-2 (COX-2) activation in intestinal crypts [65]; the number of cryptal cells expressing COX-2 was higher in AFSC treated animals and correlated with the degree of intestinal damage, and therapeutic benefits of AFSC transplantation were abolished by selective COX-2 inhibition [65].

Hematopoietic System

Murine and human CD117+/Lin− AFSC have been shown to differentiate, in vitro, into erythroid, myeloid, and lymphoid hematopoietic cells [12]. The long-term hematopoietic repopulating capacity of murine AFSC has also been demonstrated in primary and secondary transplantation experiments in irradiated mice [12]. The latter results support the idea that the amniotic fluid may be a source of stem cells with potential for therapy of hematological disorders.

One of the most significant applications of AFSC in this setting is in the field of in utero transplantation (IUT) for the prenatal treatment of congenital hematological diseases (e.g., thalassemia, sickle cell disease) [66]. IUT of bone marrow-derived HSC (BM-HSC) has been shown to have great therapeutic potential in animal models, but clinical translation has been limited by competitive and immunological barriers [67]. Such limitations could be addressed by using AFSC instead of BM-HSC for IUT, as AFSC are of fetal origin (should be able to compete with host fetal cells better than adult equivalents), and they are nonimmunogenic to the fetus and mother (due to tolerogenic properties of the placenta). IUT of AFSC would involve harvesting the cells from the amniotic fluid, in vitro gene therapy to correct the genetic defect, and transplantation back to the donor fetus. Such a combined autologous stem cell–gene transfer approach would also address some of the risks associated with administering gene therapy directly to the fetus (cells can be checked for insertional mutagenesis prior to transplantation and there would be no risk of germline transmission of transgenes) [68]. We have performed proof of principle studies in sheep, and showed that IUT of autologous, expanded, and "gene-engineered" AFSC resulted in hematopoietic engraftment in the ovine fetus [68]. We are

currently investigating the hematopoietic potential of freshly-isolated and expanded AFSC following intravenous transplantation in immuno-competent fetal mice, and have obtained stable, multi-lineage engraftment at near-therapeutic levels using relatively small donor cell numbers [55].

Musculo-Skeletal System

Our group were the first to provide data on AFSC as a promising therapeutic option for skeletal muscle degenerative diseases. We demonstrated stable and functional long-term engraftment of donor AFSC in the skeletal muscle of a murine model of human muscular dystrophy (HSA-Cre SmnF7/F7 mice) [26]. Following transplantation (tail vein injection) of 25,000 freshly isolated AFSC (without previous expansion in culture), there were significant improvements in survival rate (by 75%) as well as restoration of muscle phenotype compared to untreated controls. Transplanted mice displayed enhanced muscle strength, improved survival rate by 75% and restored muscle phenotype in comparison to untreated animals. More importantly, our work provides evidence supporting the functional integration of donor AFSC in the muscle stem cell niche; donor cells were found in sublaminal locations, expressed Pax7 and alpha-7-integrin, and could be used successfully in secondary transplantation experiments (using untreated HSA-Cre SmnF7/F7 mice as recipients). In order to progress toward their application for therapy, the therapeutic potential of cultured AFSC was also investigated and 25,000 AFSC, expanded under embryonic-type conditions, were intravenously injected into SmnF7/F7 mice. Cultured AFSC regenerated approximately 20% of the recipient muscle fibers compared to 50% when employing freshly isolated AFSC were used, highlighting the importance of optimizing cell expansion protocols.

We have also studied the potential of human AFSC to generate bone, using appropriate culture conditions (media with dexamethasone, β-glycerophosphate, and ascorbic acid-2-phosphate), and seeding these "primed" AFSC in collagen alginate scaffolds that were subsequently transplanted in immunodeficient mice. Analysis with micro-CT 18 weeks following transplantation demonstrated blocks of bone-like material [17]. Sun and colleagues confirmed the osteogenic potential of AFSC (AFSC cultured with bone morphogenetic protein-7, seeded on nanofibrous scaffolds), and demonstrated de novo bone formation in vivo using von Kossa staining and x-ray [69]. Finally, Goldberg's group compared the osteogenic potential of AFSC with that of BM-MSC in vitro [70]. AFSC and BM-HSC seeded on biodegradable polymer [poly-(ϵ-caprolactone) (PCL)] could be in cultured osteogenic media for up to 15 weeks; despite the fact that BM-MSC differentiated more rapidly than AFSC, overall production of mineralized matrix in scaffolds seeded with AFSC was five times higher due to the fact that growth and matrix production in BM-MSC cultures stopped after 5 weeks.

Nervous System

We and others have investigated the salutary effects of human AFSC in models of neuronal disease and injury. De Coppi et al. demonstrated engraftment, neuronal differentiation and prolonged survival (up to 2 months) of human AFSC transplanted directly to the brain of mice with disease features similar to those of Krabbe globoid leucodystrophy (Twitcher mice). Interestingly, AFSC survival in this setting was achieved only when transplantation was performed in diseased (but not healthy) mice [17].

Subsequent studies have not demonstrated neuronal differentiation of AFSC both in vitro and in vivo, but have shown significant beneficial effects in models of neuronal injury through paracrine mechanisms. Prasongchean and colleagues used the chick embryo model of extensive thoracic injury to demonstrate the beneficial effects of human AFSC to the nervous system [71], and similar results were obtained when rat [72], or human [73, 74] AFSC were implanted at sites of traumatic nerve injury (e.g., resection or crush injury of the sciatic nerve). Although in most studies the ultimate phenotype of donor AFSC was not examined [73–78], in a study by Pan et al. AFSC were observed around the site of injury but not penetrating into the nerve. The therapeutic effect (improved sciatic nerve motor function) was maintained despite the fact that donor AFSC did not survive for more than a month [73].

In addition to congenital and traumatic brain/nerve injury, salutary effects of AFSC transplantation have been demonstrated in models of cerebral ischemia-reperfusion injury (stroke) [78, 79]. Intracerebroventricular [78], and intravenous [79] administration of rodent AFSC following cerebral ischemia (middle cerebral artery occlusion in mouse [78] and rat [79]) and reperfusion resulted in improved stroke neurological severity score as well as cognitive and motor function up to one month after transplantation. The mechanism of action of AFSC in this setting has not been determined, although a paracrine effect mediated by release of growth factors to restore cellular function in the injured brain has been implicated [80]. To date, human AFMSC only (vs. AFSC) have been used successfully against ischemia-reperfusion injury of the brain with evidence of astrocytic preponderance of differentiation and resulting beneficial effects arising from growth factors rather than differentiated cells [81]. This is in line with research done with MSC [82, 83].

Finally, the amniotic fluid can be a source of stem cells for the in utero treatment of spina bifida and other neural tube defects. Turner and colleagues isolated "neural-type" AFSC, transplanted them prenatally in rats with experimentally induced spina bifida and demonstrated homing of donor cells at the site of injury [84]. More recently, the concept of trans-amniotic stem cell therapy (TRASCET) with AFMSC has been introduced as a minimally invasive alternative to fetal surgery for the prenatal coverage of myelomeningocele defects [85, 86]. TRASCET involves intra-amniotic administration of large numbers of AFMSC resulting in partial or complete coverage of experimental spinal bifida in the rat [85], with associated improvement in the incidence of the Arnold-Chiari malformation [86].

Respiratory System

The significant regenerative potential of AFSC has been demonstrated in the lung. AFSC are able to migrate to the lung and differentiate to specific pulmonary cell types depending on the type of injury [87]. AFSC transplanted in mice subjected to hyperoxia-induced lung injury, migrated to the lung and differentiated in surfactant protein C expressing type II pneumocytes. In contrast, following naphthalene injury to club cells (bronchiolar exocrine cells) AFSC expressed the club cell-specific 10-kDa protein [87]. Moreover, In an adult rat

model of hyperoxia lung injury, treatment with human AFSC has a reparative potential through paracrine involvement in alveolarization and angiogenesis [88]. In an established nitrofen-induced rat model of lung hypoplasia, Lung growth, bronchial motility, and innervation were rescued by AFSC both in vitro and in vivo. This is similar to what has been observed before with retinoic acid. The AFSC cell beneficial effect was probably related to paracrine action of growth factor secretion [89]. Those results have been recently validated in fetal rabbit with a surgically-created left diaphragmatic hernia at D23 (term D32). In this model, human AFSC exert an additional effect on Tracheal Occlusion leading to a decrease in mean terminal bronchiole density, a measure of alveolar number surrounding the terminal bronchioles, without signs of toxicity [90].

Urinary System

AFSC have also been shown to have significant nephrogenic potential. AFSC injected in murine embryonic kidneys and subsequently cultured ex-vivo contributed to various components of the developing kidney (including the renal vesicle S- and C-shaped bodies). Moreover, there was evidence of renal differentiation as evident by expression of the kidney markers zona occludens-1, glial-derived neurotrophic factor and claudin [91].

In a murine model of glycerol-induced acute tubular necrosis, transplanted AFSC engrafted to the injured kidney with associated improvement in creatinine and blood urea nitrogen levels and reduction in the number of damaged tubules. It has been speculated that the latter may be due to AFSC accelerating the proliferation of partially damage epithelial tubular cells, while in addition preventing apoptosis [92].

Sedrakyan et al. used a mouse model of Alport syndrome [Col4a5(−/−) mice] and demonstrated salutary effects of AFSC against renal fibrosis (delay in interstitial fibrosis and glomerular sclerosis, prolonged survival) [23]. However, AFSC were not demonstrated to differentiate into podocytes, suggesting that the positive effects to the basement membrane were mediated again, as in other model of disease, by a paracrine mechanism [36, 38, 61, 87, 88, 93, 94]. Only very recently it was reported for the first time that AFSC, mixed with organoids made with murine embryonic kidney, contributed to the formation of glomerular structures, differentiated into podocytes with slit diaphragms, and internalized exogenously-infused bovine serum albumin, attaining unprecedented, for donor stem cells, degrees of specialization and function in vivo [95].

Summary

The amniotic fluid is an under-utilized source of stem cells, with therapeutic potential in the field of regenerative medicine. Stem cells from the amniotic fluid can be isolated and expanded easily, and have the ability to differentiate into a various cell types without the risk of tumorigenesis. These cells can successfully engraft in multiple organs, and emerging evidence from experimental models of disease has generated great interest in potential clinical applications for human tissue repair and regeneration. The latter, in combination with the lack of ethical concerns associated with other stem cell sources, makes stem cells derived from the amniotic fluid prime candidates for the development of novel therapies against a wide range of congenital and acquired human disorders.

Acknowledgments

We thank Alfonso Tedeschi for his help with figures and references. S.L. is supported by the Wellcome Trust; P.D.C. is supported by the NIHR, OAK Foundation and Great Ormond Street Hospital Children's Charity.

Author Contributions

S.L.: Literature review, manuscript writing, manuscript revision, figures design. P.D.C.: Manuscript design, manuscript writing, manuscript revision.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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