Arch Pharm Res Vol 35, No 2, 271-280, 2012
DOI 10.1007/s12272-012-0207-7

Archives of Pharmacal Research
www.springer.com/12272

REVIEW

Amniotic Fluid-Derived Stem Cells in Regenerative Medicine Research

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(Received November 1, 2011/Revised December 7, 2011/Accepted December 7, 2011)

The stem cells isolated from amniotic fluid present an exciting possible contribution to the field of regenerative medicine and amniotic fluid-derived stem (AFS) cells have significant potential for research and therapeutic applications. AFS cells are multipotent, showing the ability to differentiate into cell types from all three embryonic germ layers. They express both embryonic and adult stem cell markers, expand extensively without feeder cells, double in 36 h, and are not tumorigenic. The AFS cells can be maintained for over 250 population doublings and preserve their telomere length and a normal karyotype. They differentiate easily into specific cell lineages and do not require human embryo tissue for their isolation, thus avoiding the current controversies associated with the use of human embryonic stem (ES) cells. The discovery of the AFS cells has been recent, and a great deal of work remains to be performed on the characterization and use of these cells. This review describes the various differentiated lineages that AFS cells can form and the future of these promising new stem cells in regenerative medicine research.

Key words: Amniotic fluid-derived stem (AFS) cells, Differentiation, Regeneration, Reconstruction, Tissue engineering, Regenerative medicine

INTRODUCTION

Recent progress in tissue engineering and regenerative medicine has prompted creation of functional tissues or organs *in vitro* and their successful integration into human recipients (Atala et al., 2006; Raya-Rivera et al., 2011). The objective of tissue engineering is to reconstruct tissues or organs in order to replace damaged and injured parts of the body. The major components of tissue engineering approaches are tissue-specific cells, biomaterials (also called a scaffolding system) and an appropriate environment for promoting tissue maturation/formation. A well-balanced combination of these components can allow the engineering of a tissue construct *in vitro* and further facilitate the development of functional tissue substitutes following *in vivo* implantation (Langer and Vacanti, 1993). Recently, a broad and multidisciplinary field termed "regenerative medicine" emerged through the integration of tissue engineering, stem cell biology, material sciences, developmental biology and molecular biology. The goal of regenerative medicine is to create functional tissues or organs for use in repair or replacement procedures in patients based on clinically relevant approaches (Atala, 2007). However, these cell-based approaches are often limited by the availability of an appropriate cell source and current technologies are based mostly on the use of tissue-specific primary cells taken from the patient's own body. Even though these autologous cells are non-immunogenic, they tend to be poorly proliferative and easily lose their phenotype in culture. For instance, isolated primary chondrocytes can dedifferentiate after relatively few passages. Consequently, most researchers have recently concentrated on stem cells as an alternative cell source for these cell-based approaches, because stem cells capable of differentiating to multiple lineages would be valuable for a wide range of

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therapies. In this review, we will focus on the use of one specific type of stem cells, the amniotic fluid-derived stem (AFS) cell, as a cell source in regenerative medicine research.

Amniotic fluid is known to contain multiple cell types derived from the developing fetus (Priest et al., 1978; Polgar et al., 1989). Thus, human amniotic fluid has been used in prenatal diagnosis for a number of years, and by screening this fluid and the cells contained within it, a variety of genetic and developmental disorders of the fetus can be diagnosed. When cells derived from amniotic fluid are isolated, the resultant culture consists of a heterogenous cell population displaying a range of morphologies and behaviors. Studies on these cells have characterized them into many shapes and sizes, varying from 6 to 50 mm in diameter and most range from round to squamous in shape. Most cells in the fluid are terminally differentiated along epithelial lineages, and have limited proliferation and differentiation capabilities (von Koskull et al., 1981; Medina-Gomez and Johnston, 1982). Previous studies have noted an interesting composition of the fluid, in that it consists of a heterogeneous cell population that expresses markers from all three germ cell layers (Cremer et al., 1981). Thus, many research activities have been conducted on the source of these cells and on the fluid itself. Current theories suggest that the fluid is largely derived from urine and peritoneal fluid from the fetus, as well as from some ultrafiltrate from the plasma of the mother entering through the placenta. The cells in the fluid have been shown to be overwhelmingly from the fetus, and are thought to be mostly cells sloughed off the epithelium, digestive and urinary tracts of the fetus, in addition to the amnion (Brace et al., 1989).

Our laboratory investigated the possibility of isolating a progenitor cell population from amniotic fluid. The amniotic fluid was obtained from normal fetuses using a transabdominal approach between 14 to 21 weeks of gestation. Initially, male fetuses were used to preclude the possibility of contaminating the cultures with maternal-derived cells. These studies have shown that amniotic fluid contains a novel type of stem cell which is capable of being maintained in an undifferentiated state in culture for long periods and can be induced to differentiate into many different cell types (De Coppi et al., 2007a). In this review, we describe the isolation and characterization of AFS cells as well as their ability to differentiate into multiple lineages from all three germ layers. Further, we review preliminary studies using AFS cells and their contribution to the field of regenerative medicine, including cell-based therapies and tissue engineering perspectives.

THERAPEUITC POTENTIAL OF AFS CELLS

Isolation and characterization of AFS cells

A multipotential sub-population of progenitor cells in the amniotic fluid can be isolated through positive selection for cells expressing the membrane receptor c-kit, which binds to the ligand stem cell factor (Trounson, 2007). Roughly 0.8% to 1.4% of cells in amniotic fluid have been shown to be c-kit positive in analysis by fluorescence-activated cell sorting (FACS). The progenitor cells maintain a round shape for 1 week after isolation 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, and upon reaching 80% confluency, they need a passage every 48 to 72 h. No feeder layers are required for either maintenance or expansion. These cells 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 h, with little variation with passage (De Coppi et al., 2007a).

These AFS 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 in the undifferentiated state, as well as telomerase activity even in late passages (Bryan et al., 1998). Analysis of stem cell markers shows that the AFS cells express the human embryonic stage-specific surface marker SSEA4 and the embryonic stem cell marker OCT4, both of which are typical of the undifferentiated state of ES cells; AFPS cells and also expressed mesenchymal and neuronal stem cell markers (CD29, CD44, CD73, CD90, and CD105), but they did not express SSEA1, SSEA3, CD4, CD8, CD34, CD133, C-MET, ABCG2, NCAM, BMP4, TRA1-60, or TRA1-81, to name a few (De Coppi et al., 2007a). This expression profile is of interest as it demonstrates AFS cells express some key markers of the ES cell phenotype, but not the full complement. This hints that the AFS cells are not as primitive as ES cells and yet maintain greater potential than most adult stem cells. Although the AFS cells form embryonic bodies *in vitro* that stain positive for markers of all three germ cell layers, these cells do not form teratomas *in vivo* when implanted into immunodeficient mice (Thomson et al., 1998). Lastly, the AFS cells expanded from a single cell maintain similar properties in growth and potential as the original mixed population of progenitor cells (Atala, 2009).

Multi-differentiation

AFS cells have shown multi-differentiation capabilities into tissues or organs from all three embryonic germ layers (De Coppi et al., 2007a, Atala, 2009; Fig. 1). Table I shows the chemical-based culture media conditions required to induce each differentiation pattern.

Endoderm

To induce liver-specific differentiation, AFS cells are cultured in medium containing hepatocyte growth factor (HGF), insulin, oncostatin M, dexamethasone, and fibroblast growth factor-4 (FGF-4). These AFS cells differentiate into hepatocytes, a primary parenchymal cell type in liver, as evidenced by expression of albumin, transcription factor HNF4α, c-met receptor, MDR membrane transporter and α-fetoprotein (Atala, 2009). Although specific culture components have not been identified, the potential for lung-specific differentiation was also revealed by *ex vivo* culture of human AFS cells in mouse embryonic lungs, where injected cells can integrate into the epithelium and express the early human differentiation marker, thyroid transcriptional factor 1 (TTF1; Carraro et al., 2008).

Ectoderm

AFS cells can be induced to differentiate into neurons via culture in media containing dimethyl sulfoxide

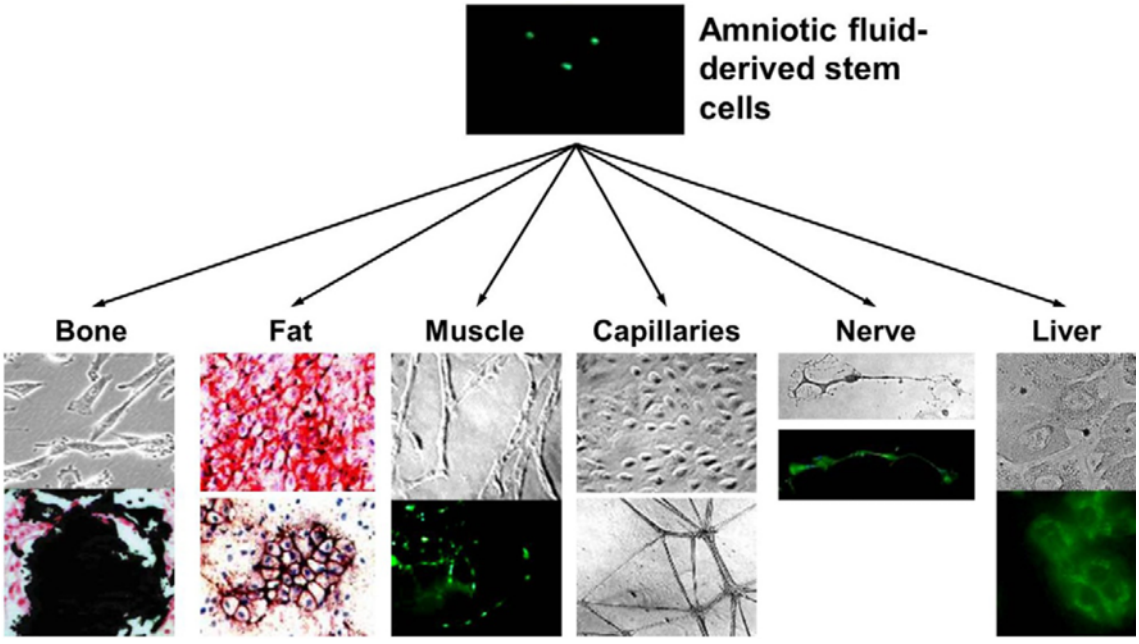

Fig. 1. Multi-lineage differentiation of AFS cells. The AFS cells have shown multi-differentiation capabilities into all three germ layer cell types.

Table I. *In vitro* AFS differentiation by chemical-based conditions (De Coppi et al., 2007a; Atala, 2009)

	Tissue-specific cell type	Culture conditions
Endoderm	Liver (hepatocytes)	Hepatocyte growth factor (HGF), insulin, oncostatin M, dexamethasone, fibroblast growth factor 4 (FGF-4)
Ectoderm	Nerve (neuronal cells)	Dimethyl sulfoxide (DMSO), butylated hydroxyanisole (BHA), nerve growth factor (NGF)
Mesoderm	Muscle (myocytes)	Pre-treatment with 5-azacytidine and horse serum and chick embryo extract on Matrigel® coated dish
	Blood vessel (endothelial cells)	Endothelial basal medium (EBM®) on gelatin coated dish
	Bone (osteocytes)	Dexamethasone, β-glycerophosphate, ascorbic acid-2-phosphate
	Fat (adipocytes)	3-isobutyl-1-methyl-xanthine (IBMX), insulin, indomethacin
	Cartilage (chondrocytes)	Dexamethasone, ascorbic acid-2-phosphate, sodium pyruvate, proline, transforming growth factor β1 (TGF-β1)

(DMSO), butylated hydroxyanisole (BHA) and neuronal growth factor. During the differentiation culture, AFS cells change their morphologies into large, flat, small and bipolar cell types, and this is followed by the appearance of cone-like terminal expansions. These neuronally-induced cells demonstrate the expression of neural-specific proteins including neuroepithelial and neuronal markers as well as some glial markers (De Coppi et al., 2007a).

Mesoderm

Myogenic differentiation can be induced by treating AFS cells with 5-azacytidine and then culturing them on Matrigel®-coated culture dishes in medium supplemented with horse serum and chick embryo extract. The differentiated cells form myotubes and express sarcomeric tropomyosin and desmin, whereas these markers are not expressed in the original progenitor population (De Coppi et al., 2007a). Adipogenic differentiation is induced in media containing 3-isobutyl-1-methyl-xanthine (IBMX), insulin and indomethacin, and was confirmed by accumulation of intracellular lipid-rich vacuoles (De Coppi et al., 2007a). Endothelial differentiation can be induced by culturing AFS cells on culture dishes coated with gelatin in endothelial basal medium, which contains epithelial growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), insulin-like growth factor 1 (IGF-1), hydrocortisone, heparin and ascorbic acid. This treatment induced expression of human-specific endothelial cell surface marker (P1H12), factor VIII (FVIII) and kinase insert domain-containing receptors as well as morphological characteristics such as cobblestone and capillary-like structures on 2 and 3 dimensional culture substrates, respectively (De Coppi et al., 2007a). Osteogenic differentiation (bone formation) can be induced by placing AFS cells in media containing dexamethasone, β-glycerophosphate and ascorbic acid-2-phosphate, and this was confirmed by observation of calcium precipitation and production of alkaline phosphatase (ALP) by the differentiated cells (De Coppi et al., 2007a). Chondrogenic differentiation can be induced by placing cells as a pellet in an alginate hydrogel and culturing the system in media containing dexamethasone, ascorbic acid-2-phosphate, sodium pyruvate, proline and transforming growth factor-β1 (TGF-β1). This differentiation was confirmed by production of sulfated glycosaminoglycan (sGAG) and type II collagen (Kolambkar et al., 2007).

Comparison with other stem cells

Table II shows the main characteristics of stem cell populations of ES cells, induced pluoripotent stem (iPS) cells, AFS cells and mesenchymal stem cells (MSC). Both ES cells and iPS cells can be difficult to differentiate efficiently and will form teratomas when injected *in vivo*. MSC are relatively difficult to expand *in vitro*. As a result, it appears that AFS cells have several advantages over these other stem cell populations. First, even without feeder cells, AFS cells have a shorter doubling time (36 h) than other stem cell populations and they can be easily differentiated into a number of cell types under specific culture conditions (De Coppi et al., 2007a). Moreover, 90% of AFS cells express the transcriptional factor Oct4, which is closely related to the maintenance of the undifferentiated state and pluripotency in ES cells (Pan et al., 2002), although AFS cells appear to be less plastic than ES and iPS cells. However, reproducibility in terms of both generation and differentiation of AFS cells has not been widely reported and further studies are required to evaluate the potential use of these promising resources. Finally, in terms of the feasibility of clinical application, AFS cells have potential for eventual use in clinical trials, since they do not induce teratoma formation *in vivo* and theoretically result in none of the ethical issues of ES cells and other stem cells. Importantly, in the pediatric field, if structural

Table II. Main characteristics of the described stem cell populations: ES, iPS, AFS and MSC

	ES cells	iPS cells	AFS cells	MSC
Source	Early stage embryo	Somatic cells	Amniotic fluid	Bone marrow and other adult tissues
Feeder cells	Required	Required	Not required	Not required
Markers	SSEA3/4, OCT-3/4, SOX2	SSEA3/4, OCT-3/4, SOX2	SSEA4, OCT4, c-kit, CD44, CD105	CD44, CD73, CD90, CD105
Plasticity	Pluripotent	Pluripotent	Broadly multipotent	Multipotent
Teratoma formation	Yes	Yes	No	No
Doubling time (h)	31-57	48	36	Variable
Lifespan *in vitro*	Long	Long	Long	Short
Ethical issues	Yes	No	No	No
Clinical trials	No	No	No	Yes

defects are diagnosed prenatally, AFS cells can be isolated by invasive sampling and cultured *in vitro* during the remainder of the pregnancy. *In vitro* expanded AFS cells could then be used to engineer a tissue construct for postnatal reconstruction of structural defects.

APPLICATIONS IN REGENERATIVE MEDICINE (TABLE III)

Muscle

In a model of acute necrotizing injury of the urinary bladder, AFS cells were transplanted for the treatment of the impaired detrusor muscle contractility resulting from the injury (De Coppi et al., 2007b). AFS cells transplanted into cryo-injured bladders formed a few

Table III. Various applications of AFS cells to regenerative medicine

	Cell types	Scaffolds	Animal model and outcomes	Refs.
Muscle	Rat AFS	n/a	Cyro-injured rat bladder walls, prevention of cryo-injury induced hypertrophy of smooth muscle cells	(De Coppi et al., 2007b)
Nerve	Neuronally –induced hAFS	n/a	*Twitcher* mice, integration with host neural cells	(De Coppi et al., 2007a)
	Rat AFS	n/a	Extensive thoracic crush injury of E2.5 chick embryo, reduction of hemorrhage and increased survival	(Prasongchean et al., 2011)
Kidney	Human AFS	n/a	Mice with glycerol-induced rhabdomyolysis and acute tubular necrosis (ATN), amelioration of ATN and decrease of damaged tubules and apoptosis	(Perin et al., 2010)
Lung	Human AFS	n/a	Mice with hyperoxia and naphthalene injury, plasticity of AFS to respond to different lung damage	(Carraro et al., 2008)
Heart	Rat AFS	n/a	Rat heart infarction by ischemia/reperfusion, improvement of ejection fraction	(Bollini et al., 2011)
	Human AFS and AFS-derived cellular structures	n/a	Heart infarction in immune-suppressed rats, improved cardiac function	(Yeh et al., 2010b, Yeh et al., 2010a, Lee et al., 2011)
Heart valve	Human AFS	Synthetic polymeric scaffold	*In vitro* formation of neo-tissues by conditioning in bioreactor system	(Weber et al., 2011)
Diaphragm	Ovine AFS	Collagen hydrogel	Partial diaphragmatic replacement of newborn lambs, mechanical and functional outcomes	(Fuchs et al., 2004)
Bone	Human AFS	Alginate/collagen	Subcutaneous implantation into immunodeficient mice, ectopic bone formation	(De Coppi et al., 2007a)
	Osteogenic differentiation of human AFS by rhBMP-7	PLLA nanofibers	Subcutaneous implantation into athymic mice, ectopic bone formation	(Sun et al., 2010)
	Rabbit AFS	PLLA nanofibers	Full-thickness sternal defects, postnatal reconstruction of chest wall	(Steigman et al., 2009)
	Human AFS	Porous PCL	Subcutaneous implantation into athymic rats, ectopic bone formation	(Peister et al., 2009)
Cartilage	Human AFS	Pellet or alginate hydrogel	*In vitro* cartilage formation	(Kolambkar et al., 2007, Kunisaki et al., 2007)
Angiogenesis	CM of human AFS	n/a	Hind-limb ischemia in mice, tissue repair by host stem cell recruitment mediated by stem cell-secreted factors	(Teodelinda et al., 2011)

rhBMP-7: recombinant human bone morphogenetic protein-7; PLLA: poly(L-lactic acid); PCL: poly(ε-caprolactone); CM: conditioned medium; n/a: non-applicable.

small smooth muscle bundles in the detrusor muscle and gave rise to limited vasculogenesis. Some AFS cells underwent cell fusion. However, it appears that the major effect of AFS cell transplantation in this model was preventing cryo-injury induced hypertrophy of the surviving smooth muscle cells via an as yet unknown paracrine mechanism.

Nerve

Our laboratory has cultured AFS cells in neuronal differentiation medium and then grafted them into the lateral cerebral ventricles of control mice and the ventricles of *twitcher* mice. The *twitcher* mouse represents a model of neurodegeneration in which a progressive loss of oligodendrocytes leads to massive demyelination and neuronal loss (De Coppi et al., 2007a). The *twitcher* mice are deficient in the lysosomal enzyme, galactocerebrosidase and undergo extensive neurodegeneration and neurological deterioration, initiating with dysfunction of oligodendrocytes which is similar to that seen in the genetic disease Krabbe globoid leukodystrophy. In our experiments, AFS cells integrated into the brains of both control and *twitcher* mice seamlessly and they appeared morphologically indistinguishable from surrounding mouse cells. In addition, they survived for at least two months post-implantation. Interestingly, more of the AFS cells integrated into the injured *twitcher* brains (70%) than into the normal brains (30%), hinting at the potential for novel therapies in diseases and injuries of the central nervous system. In this study, the phenotypes of the implanted human cells were not assessed. However, the pattern of incorporation and morphologies of cells derived from the AFS cells appeared similar to those obtained previously in the same model after implantation of murine neural progenitor and stem-like cells. In the previous case, the donor-derived cells were identified as astrocytes and oligodendrocytes.

A recent study investigated the neuronal differentiation ability of the c-kit-positive population of AFS cells isolated from green fluorescent protein (GFP)-transgenic rats and assessed how they affected injury response in avian embryos. AFS cells significantly reduced hemorrhage and increased survival when grafted at the site of an extensive thoracic crush injury in E2.5 chick embryos. This effect was mediated via paracrine mechanisms rather than the ability of AFS cells to fully differentiate into neuronal cells (Prasongchean et al., 2011).

Kidney

End stage kidney disease has reached epidemic proportions in the United States. Currently, dialysis and allogenic renal transplant remain the only treatments for the disease, but there are significant drawbacks to each. Dialysis can prolong survival by aiding renal filtration, but other kidney functions are not replaced, thus leading to long-term consequences such as anemia and malnutrition (Chazan et al., 1991). Currently, renal transplantation is the only definitive treatment that can restore the entire function of the kidney, including filtration, and production of erythropoietin and 1,25-dihydroxyvitamin D3. However, transplantation presents with several limitations, such as a critical donor shortage, complications due to chronic immunosuppressive therapy and organ failure due to rejection (Ojo et al., 2000, 2001).

Over the last decade, stem cells and their possible role in the construction of bioartificial organs, such as the kidney, have been areas of intense research. In 2007, Perin et al. showed that AFS cells could be induced to differentiate into renal cells when placed into an *in vitro* embryonic kidney environment (Perin et al., 2007). Human AFS cells were obtained from human male amniotic fluid and were labeled with Lac-Z or GFP, so that they could be tracked throughout the experiment. These labeled cells were microinjected into murine embryonic kidneys (12.5 to 18 days gestation) and maintained in a special co-culture system *in vitro* for 10 days. Using this technique, it was shown that the labeled human AFS cells remained viable throughout the experimental period and importantly, they were able to contribute to the development of various primordial kidney structures, including the renal vesicle as well as the C- and S-shaped bodies. Studies using reverse transcriptase polymerase chain reaction (RT-PCR) indicated that the implanted AFS cells began to express early kidney markers such as zona occludens-1 (ZO-1), glial derived neurotrophic factor and claudin. In addition, in later experiments, Perin et. al. also used a renal injury model in which acute tubular necrosis (ATN) caused by glycerol-induced rhabdomyolysis was induced. In this study, injected AFS cells provided a protective effect, ameliorating ATN as reflected by decreased blood urea nitrogen (BUN) and creatinine levels, in addition to a decrease in the number of damaged tubules and the amount of apoptosis. The AFS cells appeared to also have some immunomodulatory effects (Perin et al., 2010).

Together, these data suggested that AFS cells have the intrinsic ability to differentiate into a number of different cell types that make up the kidney and could represent a potentially limitless, ethically-neutral source of cells for renal tissue engineering.

Lung

Human AFS cells can integrate into murine lung and differentiate into lung-specific lineages after injury. After *in vitro* microinjection into cultured mouse embryonic lungs, AFS cells can integrate into the epithelium and express the early human differentiation marker thyroid transcription factor 1 (TTF1). In adult nude mice exposed to hyperoxia, tail vein-injected AFS cells localized in the distal lung and expressed both TTF1 and type II pneumocyte marker surfactant protein C. After naphthalene injury, specific damage to Clara cells followed by administration of AFS cells resulted in integration and differentiation of AFS cells at the bronchioalveolar and bronchial positions with expression of specific Clara cell 10-kDa protein. These results suggest a certain level of plasticity of AFS cells that allows them to respond in different ways to different types of lung damage by expressing specific alveolar versus bronchiolar epithelial cell lineage markers, depending on the type of injury to recipient lung (Carraro et al., 2008).

Heart

Although extensive cell therapy has been conducted for the treatment of myocardial infarction, a consensus is presently difficult to form and the long-term benefits of such treatments are still unknown (Menasche, 2009). As an alternative cell source, AFS cells were tested to determine whether they could differentiate into cardiac cell types following injection into a rat myocardial infarction model (Bollini et al., 2011). *In vitro*, rat AFS cells transfected to express GFP showed noticeable differentiation into myocardial cells when they were cultured with mature rat cardiomyocytes. However, no injected GFP-AFS cells were detected in injured sites in the heart following myocardial infarction, despite minimal improvement of ejection fraction in the infarcted heart. This study demonstrates that rat AFS cells have the potential to differentiate into myocardial phenotypes and improve heart function, even if their potential is limited by poor survival in an allogeneic setting (Bollini et al., 2011). Interesting approaches were reported by Yeh et al. (Yeh et al., 2010b), where various cellular structures based on AFS cells were developed *in vitro* and tested in animal models of heart infarction. By culturing AFS cells on a methylcellulose hydrogel system, the authors prepared spherical cell aggregations (Lee et al., 2011) and cell sheet fragments (Yeh et al., 2010a), and then implanted them into infarcted sites in the heart. Both cellular structures reduced cell loss after intramuscular injection and produced an enriched extracellular matrix (ECM) environment that included expression of several angiogenic and cardioprotective factors compared with dissociated AFS cells. These results suggest that AFS cell architectures would be effective for enhanced functional cardiac regeneration.

Heart valve

Using the concept of tissue engineering and regenerative medicine, several groups have demonstrated the feasibility of creating heart valves using a number of cell sources and biocompatible scaffolds. In particular, for the treatment of congenital heart diseases, prenatally harvested cells can be used to create engineered valve leaflets prior to birth. Human AFS cells were isolated and seeded on biodegradable scaffolds to reconstruct neonatal valve tissues. These tissues included viable endothelium that exhibited stable mechanical strength similar to native tissue. This study shows that it may be possible to fabricate heart valve structures *in vitro* using prenatally harvested autologous AFS cells for postnatal transplantation of the engineered tissues (Weber et al., 2011).

Diaphragm tissue

The transplantation of engineered diaphragm would be an effective long-term solution for children with congenital diaphragmatic hernia (CDH), since a number of acellular biological prostheses have not been able to provide reliable outcomes and often lead to complications such as recurrence of hernia, infection, chest wall and spinal column deformities, small bowel obstruction and restrictive pulmonary disease (Fauza et al., 2001). Ovine AFS cells were isolated and seeded on an acellular hydrogel to re-create engineered tendon structures. When the AFS-seeded tissue was implanted into a partial diaphragmatic defect in newborn lambs, the AFS-induced cellular grafts facilitated better mechanical and functional outcomes compared with acellular bioprostheses. The results demonstrated that AFS cell-seeded constructs may be a preferred method for reconstructing a diaphragmatic tissue.

Bone

From a tissue engineering perspective, the most extensive AFS cell research has been in the area of bone regeneration. Ectopic bone formation has been confirmed by subcutaneous implantation of AFS cell-seeded scaffolds *in vivo*. Osteogenically differentiated AFS cells were embedded in an alginate/collagen scaffold and implanted subcutaneously into immunodeficient mice (De Coppi et al., 2007a). By 18 weeks after implantation, highly-mineralized tissues and blocks of bone-like material were observed in the recipient mice using micro-CT. These blocks displayed a density

somewhat greater than that of mouse femoral bone. This indicated that AFS cells could be used to engineer bone grafts for the repair of bone defects.

Human AFS cells can be induced toward osteoblastic differentiation by bone morphogenic protein 7 (BMP-7; Sun et al., 2010). Nanofiber scaffolds, which mimic the morphology of natural collagen fibers, facilitated osteogenic differentiation of human AFS cells *in vitro* and bone formation *in vivo*. In addition, poly (ε-caprolactone) (PCL) biodegradable polymer was used for functional repair of large bone defects. The PCL scaffold supported the osteogenic differentiation of human AFS cells during *in vitro* culture and subsequent ectopic bone formation *in vivo*, as confirmed by micro-CT analysis and biochemical assays. This study demonstrates the potential of AFS cells to produce 3D mineralized bioengineered constructs *in vitro* and *in vivo*, suggesting that AFS cells may be an effective cell source for functional repair of large bone defects (Peister et al., 2009).

As a practical approach for postnatal sternal repair, rabbit AFS cells were isolated, cultured on biodegradable nanofibers and then implanted into full-thickness sternal defects. Two months after implantation, *in vivo* imaging modalities confirmed chest closure and bone formation. This study concluded that engineered bone tissues can be a viable alternative for sternal repair and that AFS cells can be a practical cell source for engineered chest wall reconstruction (Steigman et al., 2009).

Cartilage

Human AFS cells were expanded and cultured as a pellet within an alginate hydrogel (Kolambkar et al., 2007). To induce chondrogenic differentiation, 200,000 cells were seeded in a 15 mL conical tube in chondrogenic medium consisting of ITS (insulin, transferrin, and sodium selenite), dexamethasone, L-proline and ascorbic acid-2-phosphate. After adding growth factors such as TGF-β1, TGF-β3, BMP-2 and IGF-1, these tubes were centrifuged to condense the cells into a pellet. At 3 weeks after cell seeding, there was increased production of sGAG and type II collagen in response to TGF-β supplementation, with TGF-β1 producing a greater response than TGF-β3. Addition of IGF-1 during pellet culture further increased sGAG/DNA over TGF-β1 supplementation alone. Compared to bone marrow-derived MSC, AFS cells produced less cartilaginous matrix after 3 weeks of TGF-β1 supplementation in pellet culture. Even so, this study demonstrates that AFS cells have the potential to differentiate along the chondrogenic lineage, thus establishing the feasibility of using these cells for cartilage tissue engineering applications.

Angiogenesis

Although it is still being debated whether the effect of AFS cells described is the result of direct effects of AFS cell differentiation on functional tissue regeneration or of indirect roles as biological factor reservoirs (Caplan and Dennis, 2006); there have been several papers that describe the importance of indirect support for tissue regeneration through the secretion of biological factors by AFS cells (Yeh et al., 2010a; Lee et al., 2011). Very recently, Teodelinda et al. showed that conditioned medium (CM) from AFS cell cultures contained pro-angiogenic soluble factors, such as monocyte chemotactic protein (MCP)-1, interleukin (IL)-8, stromal-derived factor (SDF)-1 and vascular endothelial growth factor (VEGF). When injected into mice of a hind-limb ischemic model, this CM prevented capillary loss and muscle tissue necrosis, and later induced neo-arteriogenesis and remodeling of pre-existing collateral arteries. This study asserts that stem cell-secreted factors can recruit endogenous stem and progenitor cells to induce efficient tissue repair.

SUMMARY AND FUTURE DIRECTIONS

The broad types of multipotent stem cells isolated from amniotic fluid have remarkable potential in the field of regenerative medicine. Their pluripotency, high proliferation rates, multi-differentiation capability and lack of teratoma formation when injected *in vivo* make them attractive candidates for cell sourcing. In addition, there are no serious ethical issues with the use of these cells, which is an advantage over other stem cells such as ES cells and iPS cells. Very recent exciting results using AFS cells or AFS-combined engineered tissues for therapeutic applications has encouraged their use in the field of regenerative medicine in more advanced and broader manners. Although more studies remain to be performed on the characterization and use of AFS cells, initial successes have been fascinating and future experiments are sure to lead to interesting developments in regenerative medicine. Furthermore, AFS cells can be cryopreserved for future self-use. When compared with ES cells, AFS cells have many similarities: they can differentiate into all three germ layers, they express common markers and they preserve their telomere length.

However, before these cells can be translated to clinical applications, a number of experiments must still be performed. First, further characterizations of cell types that have not been investigated are necessary for scientific and therapeutic applications. Although a

few engineered tissues or organs have been created using AFS cells and biomaterial scaffolds, such as bone, cartilage, heart valve and muscle, several issues should be addressed to achieve successful outcomes during translation. These include the selection of biocompatible materials (scaffolds) in a tissue- or organ-specific manner, the method of anchoring the AFS cells to the scaffold and the provision of appropriate microenvironmental supports for cell survival, proliferation and differentiation. In the examination of injected AFS cells or implanted AFS-based engineered tissues or organs *in vivo*, the efficient integration of introduced AFS cells with the recipient system has occurred in several models, demonstrating structural and functional outcomes that highlight the true clinical potential of these cells. Much more sophisticated and focused approaches to probe the exact potential of these cells as well as full characterization of their source will be beneficial, as they will help to define realistic goals and applications for the use of these cells (Atala, 2009). Moreover, the ease of maintenance, proliferation and differentiation of AFS cells also provides great promise in other applications, including investigation into developmental pathways or drug screening.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. John Jackson and Jennifer Olson for editorial assistance with this manuscript.

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