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Amniotic Derived Stem Cells: Role and Function in Regenerative Medicine

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Abstract

Stem cells are undifferentiated biological cells that have the ability to differentiate into any specialized cells capable of cell division through mitosis. Amniotic stem cells (ASCs) are collectively a mixture of stem cells that can be obtained from amniotic fluid and tissue. Amniotic fluid is commonly called a pregnant woman's water. It is the protective liquid contained within the amniotic sac that lubricates the embryo or fetus that is generated by the maternal blood plasma, which is the liquid component that contains all of the blood derived cells in suspension. The amniotic fluid is absorbed through the fetal tissue and skin until the 20-25th week of pregnancy when the fetal skin begins to keratinize. This coincides with the fetal gut and its capacity to perform amniotic absorption.

Keywords: Amniotic stem cells; Fetal gut; Cartilage; Amniocentesis

Introduction

Amniotic stem cells (ASCs) can develop into many different types of tissues such as skin, cartilage, cardiac, nerves, muscle, and bone. Consequently, ASCs have been implicated to provide many potential medical applications, especially in organ and joint regeneration. ASCs can be extracted from the amniotic sac by a process called amniocentesis. This process can occur without harming the developing fetus, which is very important to emphasize because of the negative stigma around embryonic derived stem cells. It is believed due to media sensationalizing that all stem cells are derived from killing or deconstructing embryos, and this is simply no longer the case. Many types of stem cells have been identified that can be isolated without harming an embryo such as hematopoietic stem cells from bone marrow, unbiblical chords or even peripheral blood or stem cells isolated from adipose tissues [1].

Amniocentesis was originally a medical procedure used only in prenatal diagnosis when suspected genetic abnormalities and/or fetal infections were suspected, but recently it has also begun being used for extraction of ASCs. Recent studies have shown that amniotic fluid harbors rich sources of multi-potential mesenchymal, hematopoietic, neural, epithelial, and endothelial derived stem cells. An important benefit of using ASCs is that if they were to be used clinically to treat the same individual that they came from this procedure would sidestep the donor/recipient issue, which has so far restricted attempts at using donor-derived stem cells in medical therapies. The cells derived from amniocentesis have been used to generate heart valves, working tracheas, muscle, fat, bone, neural, liver, and cartilage tissues [1] and as such the first amniotic stem cell bank was created in the United States in Boston, Massachusetts [2].

The majority of stem cells present in amniotic fluid share multiple characteristics, which suggests that they all might have a common origin. It has been confirmed that amniotic fluid contains a heterogeneous mixture of multi-potential stem cells. By demonstrating that the stem cells could differentiate from all three germ layers, but could not form teratomas (tumors) following implantation into immunodeficient mice, the heterogeneous nature by which stem cells could develop a variety of tissue was proven. It was important to demonstrate that teratomas would not form because a teratoma is a tumor with tissue or organ components that resembles normal tissue or more than one germ layer. If teratomas were to develop this would absolutely inhibit the use of amniotic stem cells from further clinical consideration.

The germ layers are mesoderm, endoderm, and ectoderm. The ability of ASCs to generate tissue from all three tissue lines of differentiation ability makes ASCs different from embryonic stem cells, and more similar to adult stem cells found in bone, skin and adipose cells. These fetal stem cells harvested from amniotic fluid are more stable and plastic, meaning they have expanded clinical utility by their nature to develop other tissues than normal adult stem cells, which makes them much more desirable and more easily inducible to return to a pluripotential state.

Amniotic fluid must be harvested or gathered from a consenting donor either after a scheduled C-section operation, or during an amniocentesis. The amniotic fluid is then sent to an FDA approved lab where it is processed accordingly and safety checked. After processing, the fluid is cryogenically frozen and stored. Once the fluid is prepared for use it will be thawed and injected at the point of injury. Amniotic fluid is neither FDA approved or disapproved, as it is not a drug. It is considered a biologic allograft and falls under the FDA's Current Good Tissue Practice Regulations.

(www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatory/Guida nces/Tissue/UCM28522.pdf). It is not considered dangerous to the patient receiving the injection in any way, even if the patient is in no way related to the amniotic fluid donor [3]. Amniotic fluid and ASCs have many related benefits when considering them for medical use. First, these fluids and cells are immunologically privileged meaning they cause no reaction even between completely unrelated donors and recipients. Second, amniotic fluid also contains many more stem cells than present in adult bone marrow. ASCs are a sort of hybrid between embryonic and adult stem cells in terms of their plasticity and differentiation abilities. Third, amniotic fluid contains levels of hyaluronic acid, which aids in joint

lubrication and acts as a joint medication. It naturally occurs in all of our joints already, but giving a joint an added boost of hyaluronic acid could provide improved functionality in terms of support and also pain relief. Fourth, amniotic fluid also contains a full complement of growth factors that function to stimulate stem cells to differentiate into many different cell types. This is significant because if you inject stem cells near a knee with degenerating cartilage for example, a portion of these stem cells may attach to the damaged area and begin to grow new healthy cartilage cells supporting and healing previously untreatable injuries.

Amniotic fluid contains anti-inflammatory and anti-adhesion properties, which work wonders to heal joint, muscular, or skeletal injuries. Keeping inflammation at rest is a great way to accelerate the healing process of injuries-comparable to the procedure using ice as an anti-inflammatory agent. Anti-adhesion properties give amniotic fluid and

ASCs the ability to prevent scar tissue from forming and further damaging affected areas. These additional anti-adhesion qualities could also be essential in spinal cord repair, and in cosmetic uses where scarring becomes a problem [3].

Amniotic fluid is by nature anti-microbial and is able to prevent and eliminate potential infectious agents. This is highly desirable to aid non-healing wounds, and ASCs also protect infected areas from sustained or systemic development of infection. The use of amniotic fluid and ASCs shows promise for many medical procedures such as arthritis and even rheumatoid arthritis as ASCs contain immunomodulating compounds that can restore joint components and functions as well as fight auto-immune diseases by modulating immune system cells and associated immune potentiating agents. ASCs also show potential for treating soft tissue injuries such as those that involve tendon, ligament, and cartilage tears or more structural damages. ASCs would be able to locate damaged areas and in doing so they would be able differentiate into those cell types that are involved in order to start the healing process of tissues that typically cannot be healed without regenerative or physical medicine. ASCs also have shown clinical ability to heal wounds that were previously identified as non-healing before the fluid was administered. In a small trial of 20 foot or ankle wounds that were previously non-healing and unresponsive to previous treatment; 90% of the tested subjects healed within 3 months of amniotic fluid and ASC administration and none of the injuries required amputation of the foot or ankle. Similar injuries that do not receive the same amniotic fluid treatment often never fully healed or had been amputated to stop the infection [3].

Discovery

ASCs were first isolated from amniotic fluid in 2007. In 2010 researchers converted ASCs into pluripotential stem cells, which are very similar to embryonic stem cells without the controversy of harming or injuring embryos. The only real difference between ASCs and embryonic stem cells is that the ASCs "remember" where they came from in terms of their tissue site of origin meaning the ASCs can fully function related to their pluripotency. Outside of the womb in order to make ASCs pluripotent they must first be induced with cytokines and growth factors. This allows ASCs to differentiate into more differentiated cell types than adult stem cells, but it appears still less than embryonic stem cells [4]. In a sense, ASCs are hybrids of embryonic and adult stem cells meaning their ability to differentiate into tissues is somewhere in between these other types of stem cells, and therefore their abilities to heal will also fall somewhere in between. ASCs seem to be different both in location and in cellular construction than adult and embryonic stem cells, which means that they are new novel cells to be researched and studied extensively for human medical purposes [5].

Stem cells are divided into pluripotent cells meaning that they can differentiate into all derivatives of the primary germ layers, and multipotent meaning that they are able to give rise to many types of cells within a certain germ layer. They are also further divided into either embryonic or adult stem cells. Embryonic cells derive from the inner cell mass of a blastocyst, and because of their plasticity and unlimited capacity for self-renewal they have been proposed as potential treatments for dozens of human disorders, but as of this date no treatment has been derived from embryonic stem cells and they are largely surrounded in controversy from the means of obtaining them. Adult stem cells reside in specific locations known as niches of the human body where they are able to maintain multipotency. Their role physiologically is renewing and repopulating tissues in which they reside. Adult stem cells have been located in almost all organs and tissues other than the gonads. Despite having limited differentiation potential and proliferative capacities these adult stem cells represent possible resources for research and medicine. Adult stem cells can also be easily obtained without the controversy of embryo destruction [5].

Attempts to surpass the limitations of adult stem cells and to reach the potential of embryonic stem cells without embryos have long been sought. Using these methods of changing and modifying adult stem cells from a singular patient into pluripotential stem cells have now been accomplished. These are problematic though as they only work for the one patient and require complex techniques such as retroviral transduction of defined transcription factors. At the end of processing, adult stem cells are molecularly and functionally indistinguishable from regular embryonic stem cells. They are able to generate germ line competent cell progeny and importantly they do not form tumors when injected into mice [6]. This expensive patient specific process has been rapidly overshadowed by fetal stem cells derived from amniotic fluid. These new ASCs are more lineage committed than embryonic stem cells as they remember where they came from, but they show better lineage proliferation and differentiation capabilities than adult stem cells that have been modified.

Importantly hematopoietic, mesenchymal, neural, pancreatic, and lung precursor stem cells have been obtained from ASCs. Other areas have been the target of stem cell treasure hunts such as fetal tissue, umbilical cord blood, placenta, and fetal membranes. Umbilical cord blood is now a well-established source of transplantable hematopoietic stem cells that have better proliferative and differentiation capabilities than those taken from bone marrow. Umbilical cord blood has also recently been shown to contain mesenchymal and multipotent stem cells with embryonic stem cell like characteristics [6].

Placenta and Fetal Tissue Sources of Stem Cells

Both the placenta and fetal membranes have also been investigated for their capacity to produce stem cells. Researchers have found several cell populations with multilineage differentiation abilities as well as immunomodulatory properties, but they are present in small amounts. Subsequently studies using amniotic fluid were conducted finding the same cell types that were found in the placenta and fetal membranes, but in much larger concentrations. These cells are now identified as human amniotic fluid mesenchymal stem cells (AFMSCs) and

amniotic fluid stem cells (AFSCs). Easy access to amniotic fluid in caparison to other embryonic tissues means that these cells may hold much promise in the future of regenerative medicine [6].

Amniotic Fluid

Amniotic fluid is the clear watery looking liquid surrounding a growing fetus within the amniotic cavity. It allows the fetus to freely grow and to move inside the uterus. A large part of this growth is from the stem cells contained in the amniotic fluid. Amniotic fluid begins to appear at week 2 of gestation as only a small film of liquid; from there it progresses in volume to around 1000mL by week 34 of pregnancy. During the first half of gestation amniotic fluid is produced by active sodium and chloride transport and non-keratinized fetal skin. This allows concomitant passive transport of water. The second half of gestation amniotic fluid is made of fetal urine, gastrointestinal excretions, respiratory secretions, and substances exchanged through the sac membranes. Fluid is primarily composed of water and electrolytes, but also contains sugars, proteins, lipids, hormones, and enzymes [5]. Amniotic fluid cells derived stem cells (AFDSCs) from extra-embryonic structures and embryonic/fetal tissues are also present. ASCs are known to express the cell markers of all three germ layers, but their exact origin still seems to play a role in how they act later in later stages of differentiation. AFDSCs reach the outer area of the fluid by shedding from the amniotic cavity of developing skin, respiratory apparatus, urinary, and gastrointestinal tracts. The cells are thought to shed off of the embryo and then become lost in the amniotic fluid sea as partially undifferentiated stem cells [5].

AFDSCs display many morphologies and behaviors. They vary with gestational age and fetal development. In most conditions the amount of amniotic fluid cells increases as gestation advances. If a fetal disease is present amniotic fluid cell counts can be very reduced or abnormally elevated. Based on morphological growth characteristics the amniotic cells are classified into three different groups: epithelioid (33.7%), amniotic derived (60.8%), and fibroblastic (5.5%). Fetal abnormalities can cause other cell types to be found in the amniotic fluid such as neural cells in fetuses with neural tube defects and peritoneal cells in the case of wall malformation. The majority of cells found in amniotic fluid is terminally differentiated and have limited proliferative capabilities, but it has been demonstrated that there are subsets of cells present that harbor proliferative and differentiation potentials. First, hematopoietic progenitor cells were discovered in amniotic fluid and they can be collected before week 12 of gestation. Scientists were able to differentiate amniotic fluid cells into myocytes, which suggests the presence of nonhematopoietic stem cells in amniotic fluid [5]. This serves as the foundation of amniotic stem cell based research.

Mesenchymal stem cells (MSCs) are part of amniotic stem cells that are multipotent for mesodermal derived lineages. These cells are represented as adipose, chondrocytes, and cells that are myogenic and osteogenic in nature. These cells were initially found in adult bone marrow, but represent only 0.001% of all nucleated cells. They have been rediscovered in much higher concentration in amniotic fluid, thus significant for possible therapeutic uses. MSCs exhibit the potential to repair and regenerate damaged tissues such as muscle, tendons, and ligaments. Importantly, they also possess immunomodulatory properties meaning that they would be good at fighting diseases such as rheumatoid arthritis. This presence of a sub-population of amniotic cells that have mesenchymal abilities and at the same time possess the ability to proliferate rapidly demonstrates that amniotic fluid can be an abundant source of fetal cells that exhibit favored phenotypes and multilineage differentiation potentials. It is thought that these mesenchymal cells found in amniotic fluid will be very similar to those stem cells found in bone marrow. The official name for these cells is amniotic fluid derived mesenchymal stem cells (AFMSC) [6].

AFMSC are easily obtained in humans either in small volumes of 2 mL to 5 mL of second trimester amniotic fluid when the percentage of AFMSC present is expected to be around 0.9% to 1.5% of total amniotic fluid stem cells. AFMSC can be grown in basic medium containing fetal bovine serum (20%) and fibroblast growth factor at (5 ng/mL). It has been shown that AFMSC can also be cultured in the absence of animal serum without losing their properties, which is a fundamental finding for beginning clinical trials in human subjects [6].

ASCs show a uniform spindle-shaped fibroblast-like morphology and are able to grow rapidly in culture. An aliquot of human ASCs derived from 2 mL of amniotic fluid can increase to 180 million cells within 4 weeks of growth. The average doubling time of these cells is 25-38 hours where other stem cells isolated from bone marrow express doubling times of around 30-90 hours; meaning that should amniotic cells be used for treatment and regeneration of tissue they would work faster than other stem cell counterparts. The analysis of ASCs' transcript showed that the gene profile is stable between passages of culture, as well as endures cryopreservation and thawing well. ASCs share core sets of gene expression such as extracellular matrix remodeling, cytoskeletal organization, chemokine regulation, plasmin activation, and transforming growth factorbeta (TGF-β) and Wnt signaling pathways with other stem cells. ASCs show unique expression consisting of up-regulation of genes involved in signal transduction pathways and uterine maturation/contraction. This suggests that ASCs have a role in modulating interactions between the fetus and the uterus during pregnancy [6].

Markers	Antigen	CD no.	Roubelakis et al. (2007)	Tsai et al. (2004)	In 't Anker et al. (2003)
Mesenchymal	SH2, SH3, SH4	CD73	+	+	+
	Thy I	CD90	+	+	+
	Endoglin	CD105	+	+	+
	SBIO/ALCAM	CD166	+	nt	+
Endothelial and haematopoietic	LCA	CD14	—	nt	—
	gp105-120	CD34	—	—	—

	LPS-R	CD45	—	—	—
	Prorninin-1	CD133	—	nt	nt
Integrins	β1-integrin	CD29	+	+	nt
	α4-integrin	CD49d	—	nt	—
	α5-integrin	CD49e	+	at	+
	LFA-1	CD1Ia	+	nt	—
Selectins	E-selectin	CD62E	+	nt	—
	P-selectin	CD62P	+	nt	—
Ig superfamily	PECAM-1	CD31	+	—	—
	ICAM-1	CD54	+	nt	+
	ICAM-3	CD50	—	at	—
	VCAM-1	CD106	+	nt	—
	HCAM-1	CD44	+	+	+
MHC	I (HLA-ABC)	none	+	+	+
	II (HLA-DR, DP, DQ)	none	nt	—	—
nt-not tested					

Table 1: Immunophenotype of culture-expanded second-trimester human AFMSc: Results by different groups.

The cell surface profile of AFMSCs has been determined by flow cytometry illustrated in Table 1 above. Cultured cells are positive for mesenchymal markers, adhesion molecules, and antigens belonging to the major histocompatibility complex I. These cells were negative for hematopoietic and endothelial surface markers. AFMSCs show very broad differentiation ability in mesenchymal lineages, as well as adipose, chondrocytes, and osteocytes [5].

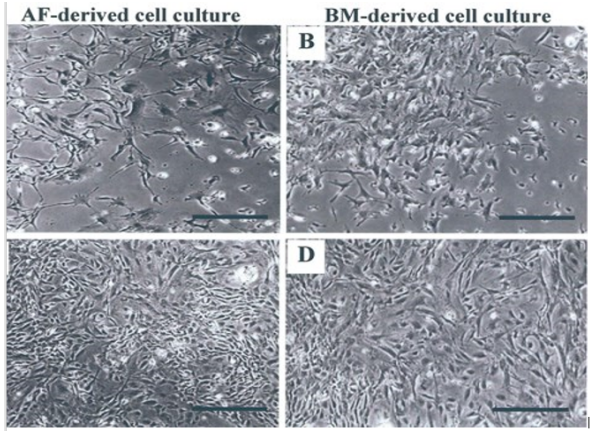

Figure 1: Photomicrograph ASCs-marrow derived stem cell cultures.

In Figure 1 the panels demonstrate the *in vitro* culture characteristics of AFSCs. This culture shows both AFSCs in A and C, and bone marrow derived stem cells in B and D. The top images were taken at 10 days after initiation for the amniotic cells and 15 days after initiation for the bone marrow cells. The cells in the images are thought to be at the same point of growth; although the amniotic cells were able to reach this point must faster. In both images, C and D fibroblastic cells have dominated the cell culture, but in the amniotic image you can see that there are more cells in the space than in the bone marrow image. In these images the bar represents a size of 100 micrometers [7].

The images A, C, and E are ASCs, while B, D, and F are bone marrow cells (Figure 2). A and B are osteogenic differentiated cultures that have been stained by alizarin red, images C and D are adipogenic cultures of cells stained by oil red, and images E and F are sections from chondrogenic micromass cultures that have been stained by toluidine blue. The bars in the images represent a length of 50 micrometers. In these images, you can see that the ASCs are normally larger or more numerous than the bone marrow derived cells, which indicates that they are growing and differentiating more quickly. This is what is desired for treatments as it would shorten patient healing times, and also may reduce number of required treatments [7].

Preclinical studies have investigated therapeutic potential of AFMSCs. In the case of a diaphragmatic hernia, repair of the muscle deficit using grafts engineered from AFMSCs leads to better structural and functional results in comparison to using fetal myoblast-based and acellular grafts. It was also demonstrated that human AFMSCs can express cardiac-specific genes under specific culture conditions. They are also able to integrate into normal or ischemic cardiac tissue where they become cardiomyocyte-like cells. In rat bladder cyro-injuries AFMSCs show the ability to differentiate into smooth muscle and prevent the compensatory hypertrophy of the surviving smooth muscle cells. It has been suggested that AFMSCs can harbor trophic and protective effects in the central and peripheral nervous systems.

AFMSCs can facilitate peripheral nerve regeneration after injury; this could be due to cell excretion of neurotrophic factors.

When AFMSCs were transplanted into the striatum they can survive and integrate in the adult rat brain where they migrate towards areas of ischemic damage. Intra-ventricular injection of AFMSCs in mice with focal cerebral ischemic reperfusion injuries significantly reverses neurological deficits in the treated mice [5]. It has been demonstrated that AFMSCs have an immunosuppressive effect *in vitro* similar to bone marrow derived MSCs. After stimulation of peripheral blood mononuclear cells with anti-CD3, anti-CD28, or phytohemagglutinin irradiated AFMSCs determine a significant inhibition of T-cell proliferation [5].

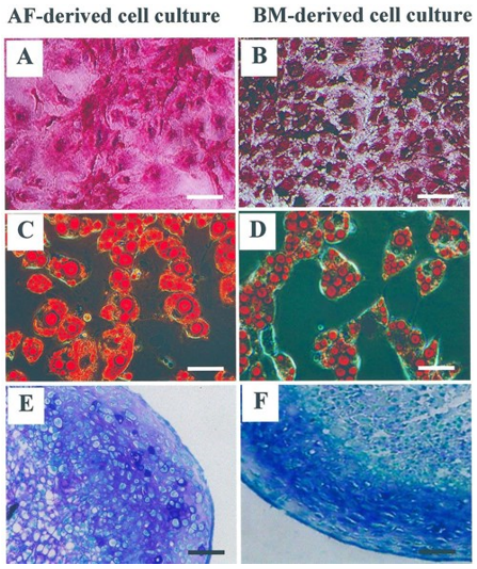

Figure 2: Differentiation potential of AF/BM derived stem cells.

ASCs are pluripotential and express the pluripotency marker Oct4 at transcriptional and proteins levels. They encompass 0.1% to 0.5% of fluid cells. Oct4 (octamer binding transcription factor 4) is a nuclear transcription factor that maintains embryonic stem cell differentiation potential and ability for self-renewal. Oct4 can be found also in ASCs, indicating that they may be just as resourceful as their embryonic counterparts. Oct4 is expressed by germ cells, and inactivation results in apoptosis. Scientists were able to transfect fluorescent protein to the Oct4 promoter meaning that they were able to identify which cells in amniotic fluid possessed these promoters and were actively using them. The only cells that were able to transcribe the promoter were the ASCs. They are characterized by expression of surface antigen c-kit (CD117), which is the type III receptor tyrosine kinase of stem cell factor [6].

AFSCs can be isolated from amniotic fluid of both humans and rodents without harming the fetus. Human cells can be derived in small volumes of 5 mL from second trimester amniotic fluid. This means somewhere between 14-20 weeks of gestation. ASCs are present during all three trimesters of pregnancy. The first trimester is the only time hematopoietic stem cells will be detected, but at this time there is so little amniotic fluid that taking even a small amount is dangerous to the embryo or fetus. The second trimester is when the amount of amniotic fluid peaks around 1000 mL and contains the highest number of ASCs. The second trimester would be the safest time to extract amniotic fluid because this is the time in which the fluid is enriched with ASCs. The third trimester also has high levels of amniotic fluid, but during this time it begins to decrease from the peak observed in the second trimester as the fetus grows larger. The third trimester also has a steadily decreasing amount of stem cell content in the fluid as the fetus is in its highest rate of growth and development.

In mice ASCs are obtainable from the amniotic fluid collected during week 2 of pregnancy. Isolation of AFDSCs is based on a two-step protocol. This protocol is carried out by FDA regulated facilities or laboratories where c-kit positive cells (approximately 1% of cells) are immunologically selected from the amniotic fluid. After extraction following identification from c-kit expression, the AFDSCs are expanded in feeder layer free, serum-rich conditions without any evidence of spontaneous differentiation *in vitro*. The cells are finally cultured in basic media containing 15% fetal bovine serum and chang supplement [5].

When ACSs are cultivated they display a spectrum of morphologies. They can appear as a fibroblast-like cell or even appear in an oval-round shape. These cells present great clonogenic potential. Clonal cell lines expand rapidly in culture with a cell doubling time of 36 hours. The more interesting statistic about clonal amniotic derived stem cell division is that they maintain constant telomere length throughout the dividing process (20 Kbp). This means they show no evidence of aging or any indication of slowing down growth over time. Despite a high rate of proliferation clonal cells show homogenous diploid DNA content with no evidence of chromosomal rearrangement even after expansion to 250 population doublings. Almost all lines of clonal cells express pluripotential undifferentiated states with Oct4 and NANOG markers. Importantly, it has been shown that ASCs do not form tumors when injected into severe immunodeficient mice [5].

The antigen profile of ASCs has been determined with flow cytometry and is illustrated graphically in Figure 2. Cultured human cells are positive for embryonic stem cell and mesenchymal stem cell markers, adhesion molecules, and antigens belonging to the histocompatibility complex I. They are negative for hematopoietic and endothelial cell markers. The unique thing about ASCs is that they are able to differentiate into tissues that represent all three embryonic germ layers of mesoderm, endoderm, and ectoderm. In mesenchymal differentiation conditions ASCs are able to form adipose, bone, muscle, and endothelial cells. When placed in adipogenic, chondrogenic, or osteogenic mediums ASCs develop intracellular lipid droplets, secrete glycosaminoglycan, and produce mineralized calcium respectively. Additionally, when ASCs are embedded in collagen scaffolds and implanted into tissues, human ASCs are able to generate blocks of bone-like mineralized tissue over 18 weeks. When ASCs are put in an environment that induces hepatocyte lineage, ASCs express hepatocyte-specific transcripts and also acquire the liver-specific function of urea secretion. In neuronal conditions ASCs are capable of entering the neuroectodermal lineage, and after induction they express neuronal markers like GIRK potassium channels and release of glutamate after stimulation. Ongoing studies of ASCs show that they are capable of forming mature and functional neurons, thus the future is optimistic for clinical use of ASCs in neurological conditions [5].

Use of ASCs in preclinical studies has begun where their ability to integrate into different tissues has been tested. In cardiovascular inducing conditions human ASCs express cardiomyocyte, endothelial, and smooth muscle markers. Although when they were xenotransplanted into rats with myocardial issues the human ASCs

were impaired by rat cell immune rejection. ASCs also have been tested in kidney development; they were injected into a mouse embryonic kidney, integrated into the renal tissue, and participated in all the steps of nephrogenesis. They express molecular markers of early kidney differentiation such as claudin or ZO-1. ASCs have also shown ability to differentiate into lung and pulmonary lineages. ASCs injected into mouse embryo lungs engraft into the epithelium and mesenchyme. They express the early pulmonary differentiation marker TFF1. In the absence of lung damage ASCs that have been administered travel to the lung, but do not differentiate into specialized cells. When lung damage is present ASCs exhibit both strong lung engraftment and express specific epithelial markers of alveoli and bronchi. Long-term experiments confirmed absence of tumor formations in treated animals up to 7 months after ASC injection [5] (Table 2).

Markers	Antigen	CD no.	De Coppi et al. (2007b)	Kim et al. (2007b)	Thai et al. (2006)
ESC	SSEA-3	none	-	+	nt
	SSEA-4	none	+	+	nt
	Tnt-I-60	none	-	+	nt
	Tra-1-81	none	-	nt	nt
Mcscnehymttl	SH2, SH3, SH4	CD73	+	nt	+
	Thyl	CD90	+	nt	+
	Endoglin	CD105	+	nt	4
Endothelial and haematopoietic	LCA	CD14	nt	nt	-
	gp105-120	CD34	-	nt	-
	LPS-R	CD45	-	nt	in
	Prominin-1	CD133	-	nt	nt
Integrins	β1-integrin	CD29	+	nt	+
Ig superfamily	PECAM-I	CD31	nt	+	nt
	ICAM-1	CDS4	nt	+	nt
	VCAM-1	CD106	nt	+	nt
	HCAM-1	CD44	4	+	÷
MHC	I (KLA-ABC)	none	+	+	+
	11 (HLA-DR, DP, DQ)	none	-	-	-
nt=not tested					

Table 2: Surface markers expressed by human AFS cells: Results by different groups.

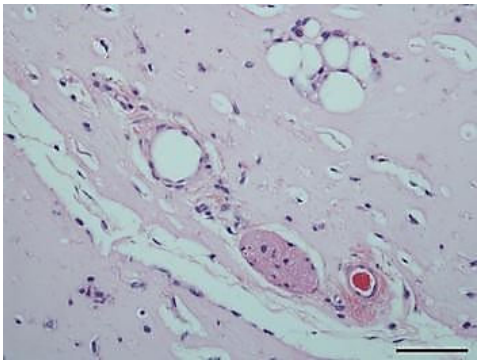

Figure 3: Growth of mature blood vessel in mouse from amniotic stem cells.

Figure 3 is a microscope image of mature blood vessels that formed in hydrogel after two weeks of growth in a mouse. There are red blood cells flowing through the vessel at the bottom right [8]. The vessels form from stem cells derived from amniotic fluid in a technique used by a Rice University lab and the Texas Children's Hospital. The scale bar represents 100 micrometers in length. This result from ASCs is groundbreaking in understanding their ability to develop new functional tissues and to also demonstrate how powerful a treatment ASCs can be. ASCs are also able to grow tissues in the laboratory setting. The process combines ASCs with a hydrogel made from polyethylene glycol and fibrin. Fibrin is a critical component for blood clotting, cellular matrix interaction, wound healing, and angiogenesis biopolymer. Angiogenesis is the process by which new vessels branch off from existing ones. Fibrin is used as a bioscaffold, but suffers from low mechanical stiffness and rapid degradation. Combining fibrin and polyethylene glycol makes the hydrogel much stronger and useable. *In vitro* vascular endothelial growth factor can prompt ASCs to become endothelial cells and the presence of fibrin encourages infiltration of vasculature from neighboring tissues. Mice injected with fibrin-only hydrogels showed development of thin fibril structures, while those injected with fibrin and ASCs showed far more robust vasculature. This technology will allow ASCs to create biocompatible patches for the hearts of infants born with birth defects and for use in other procedures [8].

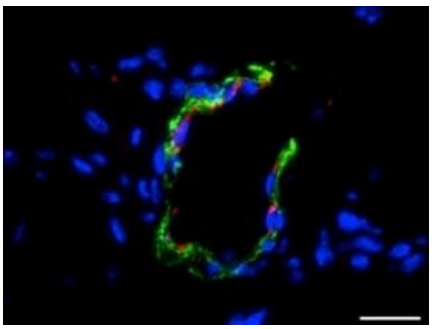

Figure 4: Hydrogel seeded amniotic fluid stem cells-Grows into a mature vessel.

A hydrogel that has been seeded with AFDSCs shows growth of mature blood vessels two weeks after implantation (Figure 4). The cell nuclei have been fluorescently stained blue, endothelial cells stained red, and smooth muscle cells stained green. All of these cells were previously undifferentiated AFCSCs [8].

Stroke

Patients who have suffered a stroke represent a promising field of clinical applications for stem cell therapy. Stroke is one of the leading causes of death and disability across the world; approximately 750,000 people in the US suffer a stroke annually and one half of the survivors have permanent disability. This disability is largely due to lost tissue in the brain that cannot be salvaged. Therapeutic potential of stem cells has been investigated as a potential treatment for this and to date showed no adverse cell related effects after 12-18 months follow up from treatment. This stem cell transplantation also correlated with improvement on the total European Stroke Scale score. Postmortem brain findings of a phase I clinical stroke trial patient who was implanted with stem cells gave researchers data that suggests implanted stem cells are able to differentiate into neurons and survive for greater than 2 years in the human brain without deleterious effects. Patients implanted with stem cells show significant improvement during follow up periods when compared to control patients. Later evaluations showed no adverse cell-related effects on the brain or nervous system. The conclusion of the study was that data from patients with severe cerebral infarcts who had been given stem cells represented a very possible and safe therapy that was able to improve functional brain recovery [6].

ASCs are beginning to be tested as a new experimental treatment for a variety of medical conditions. Because ASCs are not regulated by the FDA and thus are not considered a drug, ASCs are not covered by insurance. This will dissuade many patients from receiving the treatment. However, clinical studies that have been conducted using

ASCs have shown they have immense value and warrant continued research to determine ASCs mechanism of differentiation. Use of ASCs has shown statistically significant improvement in therapeutic regeneration of body tissues including the brain, heart, joints, and bone. Specific diseases where ASCs have been targeted for clinical use are those that cannot be treated with traditional medicine or procedures such as cartilage degeneration or rheumatoid arthritis. Cartilage degeneration or tearing is very difficult to treat because it causes pain and there are no real drugs that can reverse and repair the processes of cartilage damage. The only option is surgery, which is invasive and guarantees no significant improvements post-operation. ASCs have demonstrated the ability to differentiate into cartilage tissues that can damage tissue in joints and enhance the repairing and healing process of body [6].

ASCs also would be beneficial for rheumatoid arthritis as amniotic fluid contains hyaluronic acid, immunomodulatory agents, and stem cells. Hyaluronic acid is a joint lubrication substance that is naturally occurring in our joints. It provides support that joints need to be able to glide over one another smoothly without causing damage to tissues involved such as cartilage, tendons, or ligaments. Immunomodulatory agents present in amniotic fluid would repeal the autoimmune disorders that cause the disease in joints. By modulating the immune system in only a single targeted area amniotic fluid would not leave a patient immunocompromised, it would only hamper the immune system in the joint where it is over-actively attacking its own tissues as an autoimmune disease. The final key to this treatment is the healing process that would occur when the stem cells flock to the damaged area. Amniotic stem cells would be able to find the damaged joints caused by rheumatoid arthritis and begin differentiation into those tissues that were being damaged whether they are bone, cartilage, tendon, ligaments, or muscle-the amniotic stem cells are able to differentiate into those types of cells [6]. These types of medical conditions in which there is no drug that is able to both lessen the symptoms and begin the healing process are prime targets for amniotic stem cell therapy. ASCs are a great solution for patients who have these conditions but wish to not undergo surgery.

Use of Amniotic Membranes

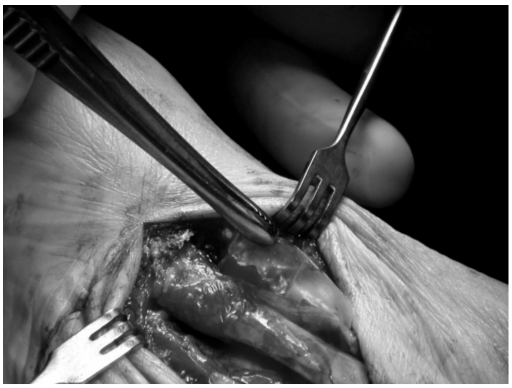

Figure 5: Application of amniotic membrane to posterior tibial tendon.

Figure 5 displays the application of amniotic membrane to posterior tibial tendon following repair in this is image an amniotic membrane is being applied to the tibial tendon after it had been surgically repaired; the membrane was applied to reduce scar tissue formation [9].

For Profit Companies Engaged in ASCs Procurement and Use Clinically

To date there are 14 companies or distributors that provide commercially available amniotic derived products. Many use proprietary processing methods and most have not made their processing methods public. Given the known effects of processing amniotic fluid on its mechanical, biological, and cellular properties a clinician would be encouraged to communicate with vendors about tissue processing before using an amniotic fluid products on patients. One common use of amniotic fluid is in ophthalmology as a treatment for corneal surface injuries. Amniotic fluid is used to form reconstructive scaffold after resection of ocular surface lesions, and also as a promoter of limbal stem cell regeneration. It is also widely used by plastic surgeons as a biological wound dressing for burns and chronic wounds. Additionally, it has been widely used as a reducer of adhesion formation after flexor tendon repairs. Podiatrists and orthopedic foot and ankle surgeons have demonstrated amniotic fluid's use in accelerating and healing of diabetic foot ulcers and non-healing post-operative wounds [10].

Clinical trials in humans have largely been experimental and have incorporated very small sample sizes, but this does not imply that these small experimental studies have not been successful. Amniotic fluid

and ASCs have been largely successful in almost all tissue regeneration and healing studies performed to date. Large clinical trials using amniotic fluid so far have only been done on other mammals such as rats, mice, and horses. In 3 studies totaling around 100 horses the use of amniotic fluid in treatment of tissue and skeletal damage showed significant results where re-injury rates were reduced and treated horses improved faster than horses that received bone-marrow derived stem cells. Healing models for tendons have also been performed. Injection of fresh amniotic fluid into a healing Achilles tendon that had been torn did not improve the histologic appearance of the tendon, but did increased collagen production and cross-linking in the healing tendon, which resulted in improved biomechanical characteristics [10].

Sports medicine is going to be one area of clinical medicine where amniotic products will be used to treat a variety of sport-related injuries in the future. Thus, it is rationale that some of the human clinical trials performed to date have been from the sports medicine field. In two randomized trials, amniotic fluid was shown to be statically significant in its ability to improve tissue damage over controls. The first clinical trial showed that amniotic fluid and ASCs injected into areas of tissue damage healed faster and better than those injected with saline. A second clinical trial demonstrated that injection of previously cryopreserved human amniotic fluid and cells had similar functional outcomes as corticosteroid injections for plantar fasciitis. No adverse effects were documented [10]. Other clinical trials are ongoing. In one amniotic fluid is being used to test how it can reduce scar formation after total knee arthroplasty. Another is assessing the effects of amniotic fluid and ASCs on zone II flexor tendon repair. A third study is comparing injecting of amniotic fluid vs. a placebo in patients with osteoarthritis [10].

If patients were to inquire whether a clinic offered ASC treatments, the number of treatments and cost could vary. Depending on the length of time the patient has had the injury or the amount of damage there is to the affected area would determine the number of treatments required. Also, if the patient has already had one or more surgeries on the same joint, for example, this will influence the amount of treatments patients would require. The size of the affected area also would indicate the number of treatments or total costs as well. For example, a very small area like one side of a hand to a simpler injury like a damaged tendon, would probably only require one injection into the hand at the affected area. This treatment would probably cost the patient between $1000$3000 dollars. Compared to patients who have damage in a much larger area such as a shoulder and have had multiple surgeries on that shoulder where scar tissue has built up would carry a higher cost of treatment. Such a patient would most likely require 2-3 treatments spaced out over 6 months, or one treatment every eight weeks. The cost of these treatments would be more as the affected area is more damaged and would require a higher volume of amniotic fluid and ASCs. Collectively these treatments could increase to $5000-$8000 per treatment in order to ensure that the damaged area has sufficient fluid and ASCs to begin the healing process and recover from injury. Final costs would depend on the overhead cost of the clinic to obtain amniotic fluid and ASCs [3].

For a specific condition such as rheumatoid arthritis it would be understandable to recommend two treatments of high dose amniotic fluid injected into the affected hands. One treatment per eight weeks would cost the patient roughly $4000-$5000 per treatment as higher volumes of amniotic fluid and ASCs are required for the affected area to begin healing damaged tissue.

Commercially the only “for-profit” companies are also the distributors of amniotic fluid products, or clinical facilities providing the stem cell administration. Each company has its own ability of obtaining and processing the amniotic fluid for safe use in patients. These distributing companies then sell to the clinicians who advertise their ability to administer stem cells as a wound healing, or therapeutic alternative to surgery and post-surgical rehabilitation. Medical practices such as physical medicine clinics or chiropractic clinics would also be for profit, as they would be charging the patient to cover the cost of the product, the administration, and operation of that medical practice [10].

There are companies beginning to perform research by first procuring amounts of amniotic fluid. A company called Regenexx is beginning to micronize amniotic fluid, which is a process in which the cells are first freeze-dried and then processed. This process destroys all of the cells, but the mixture does retain all of the extra cellular matrix, growth factors, and collagen required for optimum clinical effects. The product is officially named AmnioFix, but it would not be classified as stem cell therapy as there are no living stem cells in the product [11]. Some orthopedic clinics have begun offering full-fledged amniotic stem cell injections for joint, tendon, and ligament repair. The Hedley

Orthopedic Institute in Phoenix, Arizona offers these treatments to any and all patients.

The product that they use is called BioDFactor Viable Tissue Matrix, and it contains live ASCs. It is a liquid allograft that contains the cells, proteins, cytokines, growth factors, and other chemical compounds such as hyaluronic acid. Tissues that are taken in to make BioDFactor Viable Tissue Matrix are recovered, processed, and distributed by the FDA and AATB accredited tissue banks. The ASCs are taken after scheduled cesarean sections and are not taken from mothers who are currently carrying children through amniocentesis [12]. Another company beginning use of amniotic stem cells and tissues is MiMedx.

They are a global processor and distributor of human amniotic tissue by using the PURION process, which creates MiMedx, AmnioFix, and EpiFix products. The PURION process, a MiMedx proprietary process, dry preserves human amniotic tissue for later use, and has led to improved clinical outcomes. MiMedx obtains amniotic tissue from donated placentas, which would be discarded, from planned caesarean sections. MiMedx allografts from the PURION process can be stored at room temperature for five years without the need for refrigeration or freezing. The grafts can be used right out of the package without a thawing process [13].

A company known as the Alon Source Group has a product called ASG Fluid, which is an amniotic fluid derived product. ASG Fluid is a cryopreserved amniotic fluid structural tissue matrix derived from amniotic membrane and fluid that has been developed for clinical use as an easy to use liquid covering for areas of inflammation, soft tissue defects, enhancement of wound care, and joint tendonitis. It works by having a natural scaffold of interstitial collagens types I, II, and III that form parallel bundles providing mechanical integrity of the membrane while collagen type V and VI form filamentous connections between the interstitial collagens and basement membrane of the epithelium. ASG Fluid was developed utilizing a process micronizing the amnion products into an injectable form for clinical administration [14]. A company known as Alphatec Spine has a product for sale known as Aminoshield, which is an amniotic tissue barrier for use in wound covering and tissue protection from scarring. It comes from the

PURION disinfection process and has been shown to reduce scar formation in patients when applied post-operation [15].

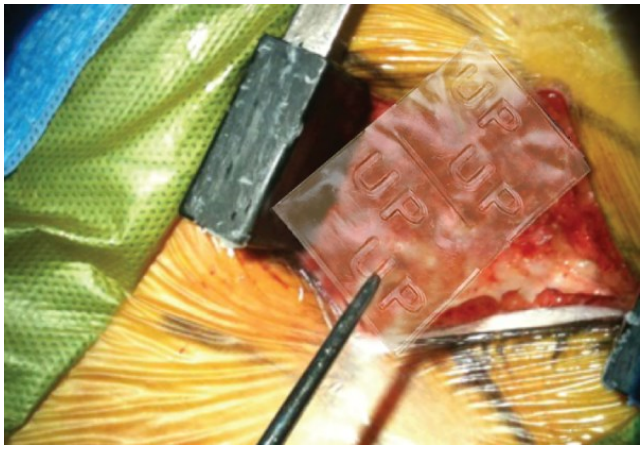

Figure 6: Amnio shield advantage being used-product of Alphatec Spine.

This product Amnioshield is a protective covering used to cover wounds that will not heal properly or to prevent the formation of scars (Figure 6). In the figure above it is being applied during a surgical procedure to reduce post laminectomy epidural adhesions. Amnioshield can come in many sizes depending on the size of the wound covering needed and is derived from amniotic tissue containing viable amniotic derived stem cells [13].

Another amniotic fluid derived product is Amniovo developed by Tri-State Biologics. Amniovo is a composite amniotic tissue membrane that is minimally manipulated to protect collagen matrix and viable cells present. Amniovo reduces scar tissue formation, inflammation, and enhances healing. It is processed using the PURION process and can be stored in regular conditions for 5 years. Amniovo is a durable graft with natural barrier abilities for optimal surgical performance. It is available in a sheet or membrane or wrap configuration for use on skin, soft tissue, tendon, and nerves [16].

Surgilogix is another company breaking into the amniotic derived product market. Their product is named AmnioFlex, which is a soft-tissue wound healing and repair that is used as an outpatient treatment. It is a liquid allograft comprised of amnion tissue and amniotic fluid, it is cryopreserved and then thawed before use. It claims to be anti-inflammatory, anti-bacterial, re-epithelializing, anti-fibrotic, and immune-privileged. Mothers donate the tissue for AmnioFlex after planned C-sections [17].

Finally, another company Surgenex provides a product called "Surforce" that contains a rich source of amniotic stem cells derived directly from amniotic membranes that are collected, harvested and purified from amniotic membranes from mothers who otherwise have normal pregnancies but undergo C-sections. The amniotic membranes that are collected serve as the source of amniotic stem cells. This amniotic membrane material has been used as implants over 100,000 times with no adverse reactions reported in patients receiving this material. SurForce generated material is regulated by the FDA as a tissue under the Public Service Act, Section 361. Every donor is required to provide a detailed medical history and is compelled to undergo a vigorous and extensive medical background screening, including testing of exposure to a number of infectious agents and related diseases [18].

The future of ASCs in terms of their eventual role in clinical medicine is complex. First to become a regular therapeutic technique extensive clinical trials in all forms of human injury are going to need to be completed and then repeated. This could take many years, but eventually doctors and the general population will accept them as a breakthrough in personalized physical medicine. Also, it will take the public to forget the many setbacks stem cells have had because of the media backlash. More than once stem cell research has been linked to dead babies and killing of embryos. The general public will never accept them as a treatment if this stigma does not go away, no matter how much your shoulder hurts nobody is going to justify killing babies if that is where they think their treatments are coming. Once ASCs are an accepted form of medical rehabilitation for complex tissue injuries throughout the body people will have much more personalized medical care and many disabled patients will be able to overcome those disabilities through the growth of new tissues. Whether that involves the regression of scar tissue over the spinal cord, and eventual spinal cord regrowth allowing previously wheelchair bound patients to walk again, or the regrowth of brain and lung tissue so that partially disabled people will regain most of the function of their old lives again. It is not known what the full extent of capabilities these cells possess, or what we can do with our knowledge of growth and development to push the cells to their absolute healing limits. Their eventual role in clinical medicine will be as an alternative way to heal. Patients may one day decide to use amniotic fluid and skip surgery and physical therapy for the healing of damaged muscle, connective, and skeletal tissue. We are only at the very cusp of understanding these new stem cells function and how best to apply them clinically. One day we will be able to use them for their specific strengths in healing providing patients with a treatment to achieve their full recovery faster and hopefully more economically than conventional therapy [6].

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