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## [17] Amniotic Fluid and Placental Stem Cells

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### Abstract

Human amniotic fluid has been used in prenatal diagnosis for more than 70 years. It has proven to be a safe, reliable, and simple screening tool for a wide variety of developmental and genetic diseases. However, there is now evidence that amniotic fluid may have more use than only as a diagnostic tool and may be the source of a powerful therapy for a multitude of congenital and adult disorders. A subset of cells found in amniotic fluid and placenta has been isolated and found to be capable of maintaining prolonged undifferentiated proliferation as well as able to differentiate into multiple tissue types encompassing the three germ layers. It is possible that in the near future, we will see the development of therapies using progenitor cells isolated from amniotic fluid and placenta for the treatment of newborns with congenital malformations as well as of adults, using cryopreserved amniotic fluid and placental stem cells. In this chapter, we describe a number of experiments that have isolated and characterized pluripotent progenitor cells from amniotic fluid and placenta. We also discuss various cell lines derived from amniotic fluid and placenta and future directions for this area of research.

### Introduction

Amniotic fluid-derived progenitor cells can be obtained from a small amount of fluid during amniocentesis, a procedure that is already often performed in many of the pregnancies in which the fetus has a congenital

abnormality. Placenta-derived stem cells can be obtained from a small biopsy of the chorionic villi. Observations on cell cultures from these two sources provide evidence that they may represent new sources for the isolation of cells with the potency to differentiate into different cell types, suggesting a new source of cells for research and treatment.

### Amniotic Fluid and Placenta in Developmental Biology

Gastrulation is a major milestone in early postimplantation development (Snow and Bennett, 1978). At about embryonic day 6.5 (E6.5), gastrulation begins in the posterior region of the embryo. Pluripotent epiblast cells are allocated to the three primary germ layers of the embryo (ectoderm, mesoderm, and endoderm) and germ cells, which are the progenitors of all fetal tissue lineages as well as the extraembryonic mesoderm of the yolk sac, amnion, and allantois (Downs and Harmann, 1997; Downs *et al.*, 2004; Gardner and Beddington, 1988; Loebel *et al.*, 2003). The latter forms the umbilical cord as well as the mesenchymal part of the labyrinthine layer in the mature chorioallantoic placenta (Downs and Harmann, 1997; Moser *et al.*, 2004; Smith *et al.*, 1994). The final positions of the fetal membranes result from the process of embryonic turning, which occurs around day 8.5 of gestation and “pulls” the amnion and yolk sac around the embryo (Kinder *et al.*, 1999; Parameswaran and Tam, 1995). The specification of tissue lineages is accomplished by the restriction of developmental potency and the activation of lineage-specific gene expression (Parameswaran and Tam, 1995; Rathjen *et al.*, 1999). This process is strongly influenced by cellular interactions and signaling (Dang *et al.*, 2002; Li *et al.*, 2004).

The amniotic sac is a tough but thin transparent pair of membranes that holds a developing embryo (and later fetus) until shortly before birth. The inner membrane, the amnion, contains the amniotic fluid and the fetus. The outer membrane, the chorion, contains the amnion and is part of the placenta (Kaviani *et al.*, 2001; Kinder *et al.*, 1999; Robinson *et al.*, 2002). Amnion is derived from ectoderm and mesoderm, which grows and begins to fill, mainly with water (Robinson *et al.*, 2002). Originally it is isotonic, containing proteins, carbohydrates, lipids and phospholipids, urea, and electrolytes. Later, urine excreted by the fetus increases its volume and changes its concentration (Bartha *et al.*, 2000; Heidari *et al.*, 1996; Sakuragawa *et al.*, 1999; Srivastava *et al.*, 1996). The fetus can breathe in the water, allowing normal growth and the development of lungs and the gastrointestinal tract. The fluid is swallowed by the fetus and passes via the fetal blood into the maternal blood. The functions of amniotic fluid are that it ensures symmetrical structure development and growth; cushions and protects the embryo; helps maintain consistent pressure and temperature; and permits freedom of

fetal movement, important for musculoskeletal development and blood flow (Baschat and Hecher, 2004).

A wide variety of different origins has been suggested for the mixture of cells within amniotic fluid (Medina-Gomez and del Valle, 1988). Cells of different embryonic/fetal origins of all three germ layers have been reported to exist in amniotic fluid (In 't Anker *et al.*, 2003; Prusa *et al.*, 2004). These cells are thought to be sloughed from the fetal amnion, skin, and alimentary, respiratory, and urogenital tracts. In addition, it has been reported that cells cultured from amniotic fluid as well as placenta provide evidence that they may represent new stem cell sources with the potency to differentiate into different cell types (Prusa and Hengstschlager, 2002). Interestingly, it has been demonstrated that a subpopulation of cells in amniotic fluid produces Oct4 mRNA, which is used to maintain pluripotency (Prusa *et al.*, 2003). Although research is at its early stages, these cells can be used to find treatments or even cures for many diseases in which irreplaceable cells are damaged.

#### Amniotic Fluid and Placenta for Cell Therapy

Ideal cells for regenerative medicine are the pluripotent stem cells, which have the capability to differentiate in stages into a huge number of different types of human cells. Amniotic fluid cells can be obtained from a small amount of fluid during amniocentesis at the second trimester, a procedure that is already often performed in many of the pregnancies in which the fetus has a congenital abnormality and to determine characteristics such as sex (Hoehn *et al.*, 1975). Kaviani and co-workers reported that “just 2 milliliters of amniotic fluid” can provide up to 20,000 cells, 80% of which are viable (Kaviani *et al.*, 2001, 2003). Because many pregnant women already undergo amniocentesis to screen for fetal abnormalities, cells can be simply isolated from the test fluid of infants with defects and saved for future use. With amniotic fluid cells, it takes 20 to 24 h to double the number of cells collected, which is faster than for umbilical cord stem cells (28 to 30 h) and bone marrow stem cells (more than 30 h) (Tsai *et al.*, 2004). This phenomenon is an important feature for urgent medical conditions. In addition, while scientists have been able to isolate and differentiate on average only 30% of mesenchymal stem cells (MSCs) extracted from a child's umbilical cord shortly after birth, the success rate for amniotic fluid-derived MSCs is close to 100% (In 't Anker *et al.*, 2003; Tsai *et al.*, 2004). Furthermore, extracting the cells from amniotic fluid bypasses the problems associated with a technique called donor–recipient HLA matching, which involves transplanting cells (Tsai *et al.*, 2004).

### Isolation and Characterization of Progenitor Cells

Amniotic fluid progenitor cells are isolated by centrifugation of amniotic fluid from amniocentesis. Chorionic villi placental cells are isolated from single villi under light microscopy. Amniotic fluid cells and placental cells are allowed to proliferate *in vitro* and are maintained in culture for 4 weeks. The culture medium consists of modified  $\alpha$ -modified Earl's medium (18% Chang medium B, 2% Chang medium C with 15% embryonic stem cell certified fetal bovine serum, antibiotics, and L-glutamine).

A pluripotential subpopulation of progenitor cells present in the amniotic fluid and placenta can be isolated through positive selection for cells expressing the membrane receptor c-kit (Fig. 1) (DeCoppi, 2001; Siddiqui and Atala, 2004). This receptor binds to the ligand stem cell factor. About 0.8 to 1.4% of cells present in amniotic fluid and placenta have been shown to be c-kit positive in analysis by fluorescence-activated cell sorting (FACS). Progenitor cells maintain a round shape for 1 week postisolation when cultured in nontreated culture dishes. In this state, they demonstrate 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, reaching 80% confluence with a need for passage every 48–72 h. No feeder layers are required either for maintenance or expansion. The progenitor cells derived show a high self-renewal capacity with >300 population doublings, far exceeding Hayflick's limit. The doubling time of the undifferentiated cells is noted to be 36 h with little variation with passages.

These cells have been shown to maintain a normal karyotype at late passages and have normal G<sub>1</sub> and G<sub>2</sub> cell cycle checkpoints. They demonstrate

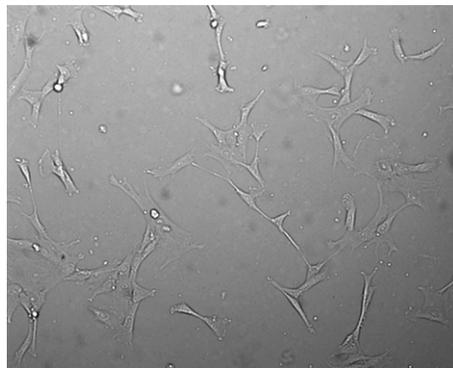


FIG. 1. Morphology of amniotic fluid-derived stem cells (AFSCs) in culture.

telomere length conservation while in the undifferentiated state as well as telomerase activity even in late passages (Bryan *et al.*, 1998). Analysis of surface markers shows that progenitor cells from amniotic fluid express human embryonic stage-specific marker SSEA4, and the stem cell marker Oct4, and did not express SSEA1, SSEA3, CD4, CD8, CD34, CD133, C-MET, ABCG2, NCAM, BMP4, TRA1-60, and TRA1-81, to name a few. This expression profile is of interest as it demonstrates expression by the amniotic fluid-derived progenitor cells of some key markers of the embryonic stem cell phenotype, but not the full complement of markers expressed by embryonic stem cells. This may indicate that the amniotic cells are not quite as primitive as embryonic cells, yet maintain greater potential than most adult stem cells. Another behavior showing similarities and differences between these amniotic fluid-derived cells and blastocyst-derived cells is that whereas the amniotic fluid progenitor cells do form embryoid bodies *in vitro*, which stain positive for markers of all three germ layers, these cells do not form teratomas *in vivo* when implanted in immunodeficient mice. Last, cells, when expanded from a single cell, maintain similar properties in growth and potential as the original mixed population of the progenitor cells.

#### Differentiation of Amniotic Fluid- and Placenta-Derived Progenitor Cells

The progenitor cells derived from amniotic fluid and placenta are pluripotent and have been shown to differentiate into osteogenic, adipogenic, myogenic, neurogenic, endothelial, and hepatic phenotypes *in vitro*. Each differentiation has been performed through proof of phenotypic and biochemical changes consistent with the differentiated tissue type of interest (Fig. 2). We discuss each set of differentiations separately.

##### *Adipocytes*

To promote adipogenic differentiation, progenitor cells can be induced in dexamethasone, 3-isobutyl-1-methylxanthine, insulin, and indomethacin. Progenitor cells cultured with adipogenic supplements change their morphology from elongated to round within 8 days. This coincides with the accumulation of intracellular droplets. After 16 days in culture, more than 95% of the cells have their cytoplasm filled with lipid-rich vacuoles. Adipogenic differentiation also demonstrates the expression of peroxisome proliferation-activated receptor  $\gamma 2$  (PPAR- $\gamma 2$ ), a transcription factor that regulates adipogenesis, and of lipoprotein lipase through reverse

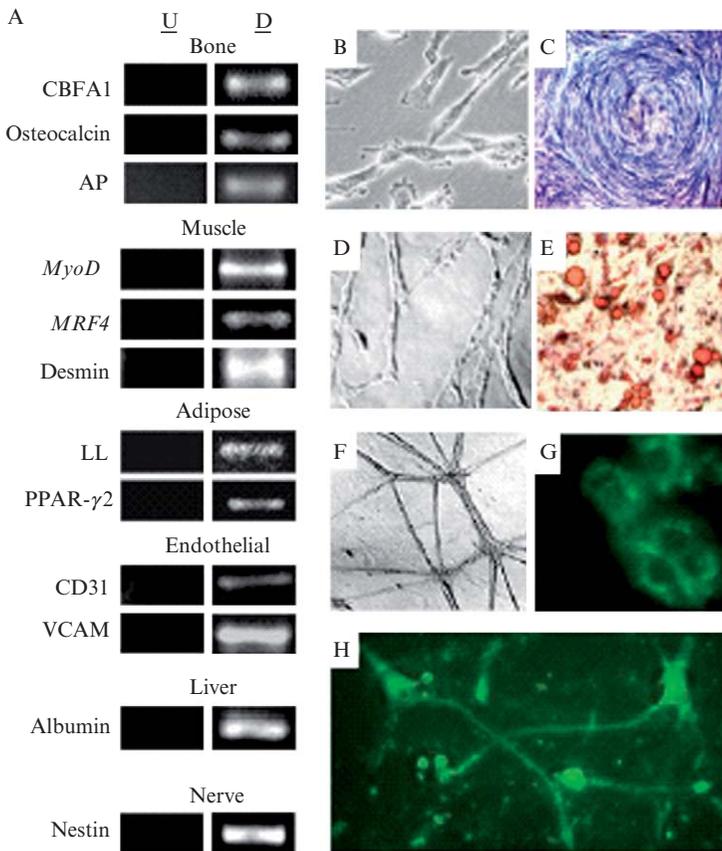


FIG. 2. Multilineage differentiation of hAFSCs. (A) RT-PCR analysis of mRNA. *Left*: Control undifferentiated cells. *Right*: Cells maintained under conditions for differentiation to bone (8 days), muscle (8 days), adipocyte (16 days), endothelial (8 days), hepatic (45 days), and neuronal (2 days) lineages. (B) Phase-contrast microscopy of control, undifferentiated cells. (b-h) Differentiated progenitor cells. (C) Bone: Histochemical staining for alkaline phosphatase. (D) Muscle: Phase-contrast microscopy showing fusion into multinucleated myotube-like cells. (E) Adipocyte: Staining with oil red O (day 8) shows intracellular oil aggregation. (F) Endothelial: Phase-contrast microscopy of capillary-like structures. (G) Hepatic: Fluorescent antibody staining (FITC, green) for albumin. (H) Neuronal: Fluorescent antibody staining of nestin (day 2).

transcription-polymerase chain reaction (RT-PCR) analysis (Cremer *et al.*, 1981; Medina-Gomez and del Valle, 1988). Expression of these genes is noted in progenitor cells under adipogenic conditions but not in undifferentiated cells.

### *Osteocytes*

Osteogenic differentiation was induced in progenitor cells with the use of dexamethasone,  $\beta$ -glycerophosphate, and ascorbic acid 2-phosphate (Jaiswal *et al.*, 1997). Progenitor cells maintained in this medium demonstrated phenotypic changes within 4 days, with a loss of spindle-shape phenotype and development of an osteoblast-like appearance with finger-like excavations into the cytoplasm. At 16 days, the cells aggregated, showing typical lamellar bone-like structures. In terms of functionality, these differentiated cells demonstrate a major feature of osteoblasts, which is to precipitate calcium. Differentiated osteoblasts from the progenitor cells are able to produce alkaline phosphatase (AP) and to deposit calcium, consistent with bone differentiation. Undifferentiated progenitor cells lacked this ability. Progenitor cells in osteogenic medium express specific genes implicated in mammalian bone development [AP, core-binding factor A1 (CBFA1), and osteocalcin] in a pattern consistent with the physiological analog. Progenitor cells grown in osteogenic medium show activation of the AP gene at each time point. Expression of CBFA1, a transcription factor specifically expressed in osteoblasts and hypertrophic chondrocytes and that regulates gene expression of structural proteins of the bone extracellular matrix, is highest in cells grown in osteogenic inducing medium on day 8 and decreases slightly on days 16, 24, and 32. Osteocalcin is expressed only in progenitor cells under osteogenic conditions at 8 days (Karsenty, 2000; Komori *et al.*, 1997).

### *Endothelial Cells*

Amniotic fluid progenitor cells can be induced to form endothelial cells by culture in endothelial basal medium on gelatin-coated dishes. Full differentiation is achieved by 1 month in culture; however, phenotypic changes are noticed within 1 week of initiation of the protocol. Human-specific endothelial cell surface marker (P1H12), factor VIII (FVIII), and KDR (kinase insert domain receptor) are specific for differentiated endothelial cells. Differentiated cells stain positively for FVIII, KDR, and P1H12. Progenitor cells do not stain for endothelial-specific markers. Amniotic fluid progenitor-derived endothelial cells, once differentiated, are able to grow in culture and form capillary-like structures *in vitro*. These cells also express platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31) and vascular cell adhesion molecule (VCAM), which are not detected in the progenitor cells on RT-PCR analysis.

### *Hepatocytes*

For hepatic differentiation, progenitor cells are seeded on Matrigel- or collagen-coated dishes at different stages and cultured in the presence of

hepatocyte growth factor, insulin, oncostatin M, dexamethasone, fibroblast growth factor 4, and monothioglycerol for 45 days (Dunn *et al.*, 1989; Schwartz *et al.*, 2002). After 7 days of the differentiation process, cells exhibit morphological changes from an elongated to a cobblestone appearance. The cells show positive staining for albumin on day 45 postdifferentiation and also express the transcription factor HNF4 $\alpha$  (hepatocyte nuclear factor 4 $\alpha$ ), the c-Met receptor, the multidrug resistance (MDR) membrane transporter, albumin, and  $\alpha$ -fetoprotein. RT-PCR analysis further supports albumin production. The maximum rate of urea production for hepatic differentiation-induced cells is upregulated to  $1.21 \times 10^3$  ng of urea per hour per cell from  $5.0 \times 10^1$  ng of urea per hour per cell for the control progenitor cell populations (Hamazaki *et al.*, 2001).

### *Myocytes*

Myogenic differentiation is induced in amniotic fluid-derived progenitor cells by culture in medium containing horse serum and chick embryo extract on a thin gel coat of Matrigel (Rosenblatt *et al.*, 1995). To initiate differentiation, the presence of 5-azacytidine in the medium for 24 h is necessary. Phenotypically, the cells can be seen to organize themselves into bundles that fuse to form multinucleated cells. These cells express sarcomeric tropomyosin and desmin, both of which are not expressed in the original progenitor population.

The development profile of cells differentiating into myogenic lineages interestingly mirrors a characteristic pattern of gene expression reflecting that seen with embryonic muscle development (Bailey *et al.*, 2001; Rohwedel *et al.*, 1994). With this protocol, *Myf6* is expressed on day 8 and suppressed on day 16. *MyoD* expression is detectable at 8 days and suppressed at 16 days in progenitor cells. Desmin expression is induced at 8 days and increases by 16 days in progenitor cells cultured in myogenic medium (Hinterberger *et al.*, 1991; Patapoutian *et al.*, 1995).

### *Neuronal Cells*

For neurogenic induction, amniotic progenitor cells are induced in dimethyl sulfoxide (DMSO), butylated hydroxyanisole, and neuronal growth factor (Black and Woodbury, 2001; Woodbury *et al.*, 2000). Progenitor cells cultured under neurogenic conditions change their morphology within the first 24 h. Two different cell populations are apparent: morphologically large flat cells and small bipolar cells. The bipolar cell cytoplasm retracts toward the nucleus, forming contracted multipolar structures. Over subsequent hours, the cells display primary and secondary branches and cone-like terminal expansions. Induced progenitor cells show a characteristic sequence of expression of neural-specific proteins. At an early stage the intermediate

filament protein nestin, which is specifically expressed in neuroepithelial stem cells, is highly expressed. Expression of  $\beta$ III-tubulin and glial fibrillary acidic protein (GFAP), markers of neuron and glial differentiation, respectively, increases over time and seems to reach a plateau at about 6 days (Guan *et al.*, 2001). Progenitor cells cultured under neurogenic conditions show the presence of the neurotransmitter glutamic acid in the collected medium. Glutamic acid is usually secreted in culture by fully differentiated neurons (Carpenter *et al.*, 2001).

## Amniotic Fluid and Placental Differentiation Protocols

### *Adipogenic Lineage Differentiation Protocol*

1. Seed cells at a density of 3000 cells/cm<sup>2</sup> on tissue culture plates.
2. Culture in low-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), penicillin–streptomycin (Pen/Strep), and adipogenic supplements [1  $\mu$ M dexamethasone, 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO), insulin (10  $\mu$ g/ml; Sigma-Aldrich), 60  $\mu$ M indomethacin (Sigma-Aldrich)].

### *Osteogenic Lineage Differentiation Protocol*

1. Seed cells at a density of 3000 cells/cm<sup>2</sup> on tissue culture plates.
2. Culture cells in low-glucose DMEM (GIBCO; Invitrogen, Carlsbad, CA) with 10% FBS (GIBCO; Invitrogen), penicillin (100 U/ml) and streptomycin (100 U/ml) (GIBCO; Invitrogen), and osteogenic supplements [100 nM dexamethasone (Sigma-Aldrich), 10 mM  $\beta$ -glycerophosphate (Sigma-Aldrich), and 0.05 mM ascorbic acid 2-phosphate (Wako Chemicals USA, Richmond, VA)].

### *Endothelial Lineage Differentiation Protocol*

1. Seed cells at a density of 3000 cells/cm<sup>2</sup> in 35-mm dishes precoated with gelatin.
2. Maintain the cells in culture for 1 month in endothelial cell medium-2 (Clonetics EGM-2; Cambrex Bio Science Walkersville, Walkersville, MD)
3. Add basic fibroblast growth factor (bFGF) at 2 ng/ml at intervals of 2 days.

### *Hepatic Lineage Differentiation Protocol*

1. Seed cells at a density of 5000 cells/cm<sup>2</sup> on plates with Matrigel (BD Biosciences Discovery Labware, Bedford, MA), using the manufacturer's thin coat method.

2. Expand in AFS growth medium for 3 days to achieve a semiconfluent density.
3. Change the medium to DMEM low-glucose formulation containing 15% FBS, 300  $\mu\text{M}$  monothioglycerol (Sigma-Aldrich), hepatocyte growth factor (20 ng/ml; Sigma-Aldrich),  $10^{-7}$  M dexamethasone (Sigma-Aldrich), FGF4 (00 ng/ml; PeproTech, Rocky Hill, NJ),  $1\times$  ITS (insulin, transferrin, selenium; Roche, Indianapolis, IN), and Pen/Strep.
4. Maintain the cells in this medium for 2 weeks, with medium changes every third day.

#### *Myogenic Lineage Differentiation Protocol*

1. Seed cells at a density of 3000 cells/cm<sup>2</sup> on tissue culture plates precoated with Matrigel (BD Biosciences Discovery Labware; incubate for 1 h at 37° at 1 mg/ml in DMEM).
2. Culture cells in basic myogenic medium consisted of DMEM low-glucose formulation containing 10% horse serum (GIBCO; Invitrogen), 0.5% chick embryo extract (GIBCO; Invitrogen), and Pen/Strep.
3. Twelve hours after seeding, add 3  $\mu\text{M}$  5-aza-2-deoxycytidine (5-azaC; Sigma-Aldrich) to the culture medium for a period of 24 h.
4. After 24 h, change the medium completely. Incubation is then continued in complete medium without 5-azaC, with medium changes every 3 days.

#### *Neurogenic Lineage Differentiation Protocol*

1. Seed cells at a concentration of 3000 cells/cm<sup>2</sup> in 100-mm tissue culture plates.
2. Culture cells in DMEM low-glucose medium, Pen/Strep, supplemented with 2% DMSO, 200  $\mu\text{M}$  butylated hydroxyanisole (BHA; Sigma-Aldrich), and nerve growth factor (NGF, 25 ng/ml).
3. After 2 days, return the cells to normal AFS growth medium lacking DMSO and BHA, but still containing NGF.
4. Add fresh NGF at intervals of 2 days.

#### Conclusion

Pluripotent progenitor cells isolated from amniotic fluid and placenta present an exciting possible contribution to the field of stem cell biology and regenerative medicine. These cells are an excellent source for research

and therapeutic applications. The ability to isolate progenitor cells during gestation may also be advantageous for babies born with congenital malformations. Furthermore, progenitor cells can be cryopreserved for future self-use. Compared with embryonic stem cells, progenitor cells isolated from amniotic fluid have many similarities: they can differentiate into all three germ layers, they express common markers, and they preserve their telomere length. However, progenitor cells isolated from amniotic fluid and placenta have considerable advantages. They easily differentiate into specific cell lineages and they avoid the current controversies associated with the use of human embryonic stem cells. The discovery of these cells has been recent, and a considerable amount of work remains to be done on the characterization and use of these cells. In future, cells derived from amniotic fluid and placenta may represent an attractive and abundant, noncontroversial source of cells for regenerative medicine.

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