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Amniotic fluid stem cell models: A tool for filling the gaps in knowledge for human genetic diseases

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Abstract:

Induced pluripotent stem (iPS) cells have attracted attention in recent years as a model of human genetic diseases. Starting from the diseased somatic cells isolated from an affected patient, iPS cells can be created and subsequently differentiated into various cell types that can be used to gain a better understanding of the disease at a cellular and molecular level. There are limitations of iPS cell generation, however, due to low efficiency, high costs, and lengthy protocols. The use of amniotic fluid stem cells (AFS) presents a worthy alternative as a stem cell source for modeling of human genetic diseases. Prenatal identification of chromosomal or Mendelian diseases may require the collection of amniotic fluid which is not only useful for the sake of diagnosis but also from this, AFS cells can be isolated and cultured. Since AFS cells show some characteristics of pluripotency, having the capacity to differentiate into various cell types derived from all three germ layers *in vitro*, they are a well-suited model for investigations regarding alterations in the molecular biology of a cell due to a specific genetic disease. This readily accessible source of stem cells can replace the necessity for generating iPS cells. Here, we expand on the applicability and importance of AFS cells as a model for discovery in the field of human genetic disease research. This paper is a review article. Referred literature in this paper has been listed in the references section. The data sets supporting the conclusions of this article are available online by searching various databases, including PubMed. Some original points in this article come from the laboratory practice in our research center and the authors' experiences.

Keywords:

Amniotic fluid stem cells, drug development model, genetic diseases, human genetic disease model, induced pluripotent stem cells

Background

Even with a growing number of investigations detailing the genetic basis for various late-onset and congenital human diseases, treatment remains out of reach for a large percentage of these conditions. The gaps in knowledge withholding development of an effective treatment typically involve preliminary molecular events in the exact order they occur during tissue development, and in some cases, the fundamentals on the pathogenesis of the disease remain unclear.

While all cells of the body may carry the chromosomal irregularity or gene mutation, the disease only presents itself in tissues expressing the abnormal gene(s) which may be limited to only a few tissues or a single tissue. Collecting cells from these damaged or abnormal tissues directly from living patients for the sake of research leads to problematic scenario, in which experiments are carried out in cells that are already damaged. By the time, the genetic disorder is diagnosed, it is likely that tissue function has already been disrupted, and therefore, the initial stages of the disease have passed, along with the chance to understand molecular events of early

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disease progression during differentiation. Animal models can be used to track these early events during development that result from a particular human genetic disorder. While these models have given valuable insights on these diseases, they are limited by key differences between species, such as anatomical and physiological variances, preventing these studies from being completely translatable to human patients.^[1] The underlying source of pathogenesis in genetic disorders is derived from the translation of genotype to phenotype and the molecular pathways interrupted by this alteration in a gene product(s); particularly, molecular pathways and their many components that may differ between species. Human stem cells present a potential model for genetic diseases that circumvents the difficulty of capturing developmental processes and variation between species, therefore, may provide the gaps necessary to comprehend the molecular basis of the disease essential for the development of an effective treatment. Human pluripotent stem cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, have been identified as the most appropriate stem cell model for genetic disease so long as they carry one of these naturally occurring mutations or genomic aberrations to be studied.^[2] Assisted reproduction technique clinics provide a source of human ES cells containing genetic mutations which can be verified by preimplantation genetic diagnosis. These naturally occurring mutated ES cells isolated from embryos can theoretically spawn any cell type in the human body.^[3] This advantage, however, has not overcome ethical concerns that were brought to light in 1998 at the time of their first successful isolation.^[4] Since then, the use of ES cells for the sake of scientific inquiry has been restricted in many regions of the world. To avoid ethical restraints, iPS cells have become a popular alternative source of pluripotent cells for the modeling of human genetic conditions. Takahashi *et al.* were the first to report conversion of somatic cells to pluripotent cells when human fibroblasts were effectively reprogrammed through the transduction of only four transcription factors.^[4] For investigators of genetic diseases, this became a revolutionary development that allowed fibroblast from afflicted patients to be isolated and transformed into pluripotent cells carrying the disease-causing genome. The formation of patient-derived iPS cells made it possible to observe early disease progression and resulting changes in phenotype once these cells were selectively differentiated into the affected lineages.^[1] The utilization of iPS cells for the modeling of genetic disease has vastly advanced our understanding of numerous disorders such as Down syndrome,^[1] Duchenne and Becker muscular dystrophy,^[1,5] Huntington disease,^[1] Gaucher's diseases,^[1,6] and Fragile X syndrome.^[7] These advancements in knowledge afforded by utilizing iPS cells often included novel insights into the

molecular mechanisms of the associated disease such as the amplified oxidative-stress response in Parkinson disease,^[1,8] the lessened synaptic connectivity in Rett syndrome,^[1] the abnormal cellular localization of KCNQ1 in LQT syndrome,^[9] and the silencing of telomerase RNA component locus in the dyskeratosis congenital disorder.^[8] Although iPS cells have been a useful tool for the progress of genetic disease research, unlike ES cells, these cells require reprogramming of adult cells through artificial means; a process that introduces several limitations. First, precisely, how similar these cells function compared to normal and disease-specific differentiated adult cells is questionable in addition to the added possibility of false negatives or positives that may be difficult to quantify. Epigenetic memory is another important limitation of iPS cells considering it may not always reflect that of a true embryonic cell.^[10] Furthermore, the fibroblast obtained from patients are typically dermal fibroblast for the sake of avoiding an invasive procedure, however, these cells are likely to carry additional mutations that have manifested over time due to consequences of aging and their exposure to the sun.^[11] This is in the context of the additional risk for gene mutations and karyotype abnormalities that may occur during propagation in culture.^[11] As a final point, iPS cells are limited to investigations of genomic aberrations or genetic mutations that are nonprenatal lethal. Overall, while iPS cells have shown their usefulness, these caveats represent the necessity for other models in the modeling of human genetic diseases to circumvent these drawbacks. Sharing various characteristics with ES cells without sharing the ethical restraints, whereas bypassing the limitations of iPS, amniotic fluid stem cells (AFS) cells make a suitable candidate for modeling of genetic diseases. This review will highlight the utilization of AFS as a worthy alternative model for research on human genetic diseases, particularly studies seeking to detail disease progression at the molecular level.

Amniotic Fluid Stem Cells: Utilization as a Research Tool and Therapy

During amniocentesis, human amniotic fluid is collected which includes a heterogeneous cell population derived from embryonic and extra-embryonic tissues. In the previous studies, AFS cells have been isolated and characterized based on their properties and gestational age.^[12] Based on morphology and growth characteristics, AFS cells have been organized into three classifications as follows: amniotic fluid specific (AF-type) cells, fibroblastic (F-type) cells, and epitheloid (E-type) cells. If present in the fluid sample, E-type cells appear late while AF-type and F-type cells consistently arise early in cultivation.^[13] In 2003, Prusa *et al.* were the first to observe an important marker of pluripotency, OCT4,

expressed by the cells suspended in human AF.^[14] Also within that year, it was reported that mesenchymal markers, such as CD73, CD90, CD105, and CD166 were observed on a fibroblast-shaped cell population derived from human AF, and these cells were negative for hematopoietic indicators, such as CD14, CD34, and CD45.^[15,16] Membrane receptor c-kit (CD117) is expressed in about 1% of cultured AFS cells,^[16] and in 2007, De Coppi *et al.* isolated c-Kit positive cell populations with high clonogenic capacity, discovering that these clonal AFS cell lines exhibit the ability to self-renew, preserve telomere length beyond 250 doublings and rapidly expand in feeder layer-free cultures at a doubling time of about 36 h.^[12] Regardless of a high proliferation rate, AFS cells are able to undergo up to 25 passages without changes in apoptosis rate, morphology, and expression of pluripotency markers.^[12,15-18] Importantly, all three germ cell layers may be derived from AFS cells *in vitro*, and under the proper conditions, may differentiate into hepatic, neural, endothelial, myogenic, osteogenic, and adipogenic cell types.^[19-23] Often described as broadly multipotent stem cells, AFS cells possess characteristics of ES cells as well as adult stem cells.^[12,15,16] A common concern with ES cell transplantation is the risk of teratoma formation, an event which was not seen in nude mice as a consequence of AFS cell administration.^[16] Moreover, unlike ES cells, ethical limitations are avoided provided that amniocentesis is the widely conventional method for the prenatal diagnosis. AFS cells themselves are modifiable through gene therapy, as they are susceptible to the first generation adenovirus vectors, whereas infection does not alter the phenotype or differentiation potential of the cell.^[24]

A number of protocols exist for the isolation and differentiation of AFS cells, most of which are based on selectivity for c-kit positive cells.^[12,15,16,25] However, some groups have chosen to allow proliferation and differentiation without the initial separation of c-kit positive from negative cells.^[26,27] When comparing c-Kit + only cultures versus unsorted AFS cells, it was shown that both groups can produce cell lineages characteristic of all three germ cell layers and show similar potential for differentiation and stemness, though overall, their properties are not identical.^[15,16,28] An extensive range of pluripotency markers are expressed by cultured human AFS cells (c-kit + and unselected), including c-MYC, KLF4, OCT4, SOX2, SSEA3, and SSEA4, in addition to various differentiation markers, such as AFP, BMP-4, GATA 4, HNF-4 α , and nestin.^[15,16,29]

AFS cells have promising potential as an allogeneic transplantation therapy for the purposes of regenerative medicine due in part to their low immunogenicity and immunomodulatory capacity. Numerous reports have shown positive expression for antigens

HLA-ABC (MHC class I) in AFS cells, with a minute population partially positive for antigens HLA-DR (MHC class II).^[12,15,16] Moreover, AFS cells have been reported to generate immunosuppressive factors such as HLA-G and protectin (CD59), therefore, decrease the chance of rejection.^[15,16] As with any type of stem cell, there has been interest in the paracrine potential of AFS cells with a secretome that consist of various elements including, vascular endothelial growth factor, stromal cell-derived factor-1 (SDF-1), monocyte chemoattractant protein-1 (MCP-1), and other important pro-angiogenic soluble factors, cytokines, and chemokines.^[30-32] *In vivo* experiments provided additional evidence to the paracrine effect of AFS cells after transplantation into the stroke rat brain resulted in a reduction in infarct volume, and a boost in endogenous cell proliferation, followed by increased differentiation into neuronal lineage.^[33,34] Even without the continuous presence of AFS cells, the isolated conditioned media alone has the capacity to produce an anti-apoptotic/pro-survival effect after acute myocardial infarction in animal models, subsequently reducing infarct size and overall cardiomyocyte death by way of pro-angiogenic and cardioprotective factors.^[35] The utilization of AFS cell conditioned media for cardiovascular disease presents a favorable therapeutic approach and can be used to identify key cardioprotective molecules.^[36]

Recently, numerous research teams have suggested that AFS cells originate from epiblast-derived cells (i.e., primordial germ cells [PGC] and PGC progenitors) due to the shared expression of c-Kit, DAZAL, fragilis, Rnf-17, Stella, and Vasa between first and second trimester human CD117+/' selected AFS cells and PGCs.^[37,38] These data guided the current theory that a number of PGCs detach during development, becoming free floating within the AF, accounting for the early germ cell markers that are present within a population of AFS cells.^[38] This running hypothesis requires further exploration and validation before the debate on AFS cell origin is closed.

Amniotic Fluid Stem Cells: Differentiation Potential

AFS cells are unique in that they are intermediate in behavior between adult and ES cells.^[15,16,39] While the previous reports have described human AFS cells as a type of pluripotent stem (hPS) cell,^[40,41,42] this is a questionable assumption considering there is no evidence to suggest chimeras can be produced from injection of AFS cells into blastocysts and injection of AFS cells does not yield tumors *in vivo*. By definition, hPS cells have the capacity to differentiate into cell lineages formed in all the three germ cell layers and may be propagated into clonal lines *in vitro*, while producing

teratomas *in vitro*; all of which supporting that AFS cells are not true hPS. Based on both molecular and biological characteristics, classification as a traditional multipotent stem cell is also a misrepresentation of AFS cells because as aforementioned, they express several indicators of pluripotency (c-MYC, KFL4, NANOG, OCT4, and SOX2) and may generate monoclonal cell lines derivative of all three germ layers. In addition, like both PGCs and ES cells, clonal human c-kit + AFS cells are capable of producing embryoid bodies (EB), a process which is regulated similarly in each cell type with the mT or pathway.^[43] Denoting a critical stage in the formation of the germ layers by way of differentiation of hPS cells, EB formation consists of three-dimensional (3D) aggregates that are evident in early mammalian embryogenesis. Moschidou *et al.* successfully produced beating EB from the first trimester human c-Kit + AFS cells with high efficiency.^[44] Thereafter, Moschidou *et al.* achieved EB formation *in vitro* with features of early stage embryogenesis and pluripotency potential and importantly, was derived from unselected second trimester AFS cells.^[45] While not an entirely equivalent replacement to true hPS cells, AFS cells hold significant value for many reasons which include easy accessibility through routine amniocentesis, capacity for EB generation, and differentiation into cell types of each germ layer, as well as their overall therapeutic safety.

Amniotic Fluid Stem Cells: A Resource for the Study and Treatment of Human Genetic Diseases

The various benefits of AFS cells over iPS cells [Table 1] allow researchers to fill in the gaps in our understanding of many human genetic disorders. Their utilization is further pushed by the rising incidence of fetuses with chromosomal aberration, a probability of occurrence that is proportional to maternal age at pregnancy which has become higher worldwide. Over time, advancements in screening techniques have developed such as nuchal translucency and detailed biochemical analysis, which can be conducted during the first trimester and permit a greater likelihood that amniocentesis will yield AFS cells containing aberrations. The isolated AFS cells can be reprogrammed using several different approaches,^[46-49]

then readily differentiated and cleared of epigenetic memory.^[10] Without using integrating or viral methods of reprogramming to avoid the risk of virally induced tumorigenicity and other complications that follow transgenes and genome integration, Moschidou *et al.* developed a protocol to reprogram first-trimester AFS cells to pluripotent cells.^[44] This technique opens up the option to utilize AFS cells as a source of pluripotent cell that may be applicable to the clinical setting. Already, second-trimester human AF-iPS cells carrying trisomy 21 have represented a useful *in vitro* model of Down syndrome. This model revealed miR-155 and miR-802-two transcripts provided by chromosome 21-as key factors contributing to deficiency in neuronal differentiation.^[50-51]

When β -thalassemia homozygous iPS cells were generated from both AFS cells and dermal fibroblasts, it was the iPS cells derived from AFS cells that outcompeted the dermal fibroblast iPS cells as determined by a doxycycline-inducible lentiviral system to evaluate efficiency.^[52] The AFS cells were reprogrammed to β -thalassemia homozygous iPS cells faster and with greater efficiency, suggesting their use as an invaluable tool in the testing of mutation-specific drugs that are potential perinatal treatments. In addition, the development of an established clonal AFS cell line for a particular human genetic disorder would be a great asset for both basic science research and drug discovery.^[53] Despite the value of AF-iPS cells, they are restricted by the range of diagnosable prenatal genetic diseases which generally excludes late-onset monogenic disorders and multifactorial diseases. Although, the newly developed technology of CRISPR/Cas9 permits genome editing, therefore, making it possible to create AF-iPS cell models of complex and monogenic genetic diseases.

Amniotic Fluid Stem Cells: A Model for Drug Development

Despite promising preclinical research and the strict qualifications required to register a drug for clinical trials, most trials fail in late phase III. These failures continue to occur in the context of vastly improved processes for generating testable compounds and advanced technologies being used to screen these compounds. The

Table 1: Comparison of embryonic stem, amniotic fluid stem, and induced pluripotent stem cell models

| Benefits | ES | AFS | iPS | Drawbacks | ES | AFS | iPS |
|--|----|-----|-----|--|----|-----|-----|
| Differentiation into 3 germ layers and pluripotent marker expression | + | + | + | Ectopic oncogene expression | - | - | + |
| Disease-specific stem cells | + | + | + | Epigenetic deregulation | - | - | + |
| Disease-specific stem cells with known patient's phenotype | - | + | + | Potential for abnormalities during reprogramming | - | - | + |
| May contain prenatally fatal mutations | + | +/- | - | Ethical controversy and legal restrictions | + | - | - |
| Naturally occurring | + | + | - | Teratoma risk | + | - | + |

ES: Embryonic stem, AFS: Amniotic fluid stem, iPS: Induced pluripotent stem, +: Indicates benefits and/or drawbacks, -: Indicates lack of benefits and/or drawbacks, +/-: Indicates weak presence of benefits and/or drawbacks

key step thought to be responsible for this high failure rate lies within the transition from animal studies to human trials. The variances in genetic makeup between species, as well as etiological and mechanistic differences in various species for a specific disease, attribute to the unpredictability of this transition. When attempting to singularize efficient treatments from a list of potential drug candidates, cell culture becomes a powerful tool. However, *in vitro* studies are limited by the applicability of the model to the actual disease occurring within the cells of the human body. For meaningful results, “physiologically-relevant cells” are imperative, making many engineered cell lines of human primary cells unfit due to their optimal biological environment and regulation from wild type, native elements. Evidently, primary cells with physiological relevancy typically display an unstable *in vitro* phenotype, a poor proliferation rate, and variability between cultures with the potential for limited accessibility as in the case with hepatocytes, neuronal cells, and pancreatic β -cells.^[53] Many of these caveats can be avoided, however, by taking advantage of iPS cell technology to produce primary cells with physiological relevance, without the limitations of poor accessibility.^[53] In regards to the use of animals for drug development, the 3Rs principle –reduce, refine, or replace – has been proposed as a way to reduce phase III failures;^[54-55] a goal that will remain out of reach without the use of *in vitro* models that properly reflect human pathology.

The utilization of iPS cells to model human genetic diseases goes beyond basic science, as many have generated these models to test the efficacy of potential drugs for a particular genetic disease. For example, iPS cells were used to sample cisapride, isoproterenol, and nifedipine for LQT syndrome,^[56] and tobramycin and valproic acid for spinal muscular atrophy.^[57] Toxicity drug screening has also been performed using iPS cells, which typically focus on hepatotoxicity and cardiotoxicity considering toxicity at these locations are a primary concern in drug safety. Human iPS cells can generate both hepatocytes and cardiomyocytes with genotypic and phenotypic features that are predicted to produce drug responses reflective of human cells *in vivo*.^[58-59] Human iPS cell has also been differentiated into neurons with characteristics similar to those occurring naturally, suggesting their use in drug screening and assessment of neurotoxicity.^[60] Given the specific overlapping properties of iPS cells and AFS cells [Table 1], it is suggested that AFS cells would also be an appropriate model for safety assays and drug discovery, although there is little evidence at this time to support this claim,^[61] albeit, it has been shown that AFS cells may be differentiated into hepatocytes and cardiomyocytes.^[62-65] There is scarce data demonstrating successful AFS cell differentiation into neurons,^[68] despite their ability to produce various neuroglial factors

and cytokines as well as induce neuroregeneration and myelination.^[59]

Several reports claim that AFS cells do however present a potential model of germ cell precursors, which are difficult to study in humans as a consequence of the timing of their formation, specifically, after implantation stage of the embryo development.^[66] An AFS cell model as a precursor for gametes would assist in the development of treatments that can prevent infertility caused by chemotherapy and other drugs without interrupting their efficacy in target cells. This model would also permit an overall advancement of our understanding of mechanisms for drug-induced complications in gametogenesis. As an example, this AFS cell model could be used to explore the reproductive hazards warranted by marijuana preparations, largely due to $\Delta 9$ tetrahydrocannabinol.^[67]

Relevant to creating novel models for drug discovery, 3D chimeric organoids are producible using AFS cells and mouse embryonic kidney cells.^[68] Importantly, the human cells can perform various functions such as form glomerular structures, differentiate into podocytes, and uptake bovine serum albumin.^[69] These 3D systems exceed the limitations of 2D cell culture, allowing for the development of organ-like structures capable of performing organ-specific functions.^[70] These organoids have been observed to sustain integral stem cell compartments, creating a unique aptitude for long-term expansion.^[71] Organoids were used in a recent study of cystic fibrosis seeking medicine specifically tailored to individual patients.^[72] Further research will be required to evaluate the value of AFS cells and organoid formation for the sake of drug discovery.

The Influence of Epigenetics and Potential for Amniotic Fluid Stem Cell Models in Epigenome Investigations

Unlike the stable nature of the genome, the epigenome undergoes various alterations influenced by the internal and external environment, and therefore epigenetic modifications occur throughout the life of an organism. Epigenetic patterns are originally established and sustained through the course of germ cell development. Any error made in the epigenome over the course of germ-line development has the capacity to affect fertility and prompt serious health deficits in potential offspring, testifying to the power the epigenome has over general well-being.^[73] Defects during epigenetic programming may be introduced with exposure to intrauterine environmental elements and may influence both the fetus and its germ line (F1 and F2 generations). Current literature suggests that direct influence by environmental factors is not the source of epigenetic modifications

passed to the F3 generations, instead, these alterations are considered to be transgenerational. While it is often still debated within the scientific community, the occurrence of transgenerational epigenetic inheritance has been reported, typically in a paternal fashion,^[74] although the precise mechanism by which this occurs remains elusive. It was in plants that biologist first observed this phenomenon,^[75] sparking interest for the sake of medical research which lead to evidence that transgenerational epigenetic inheritance also occurs in rodent and humans. The idea of transgenerational effects in humans was first introduced with epidemiological studies that were conducted in Europe at the beginning of the 21st century which presented evidence over a large population.^[76] To gain a better understanding of this occurrence, researchers have focused their interests on the mechanism behind the addition and loss of epigenetic markers in sperm due to the paternal nature of transgenerational epigenetic inheritance. Further supporting epidemiological investigations, *in vivo* experiments provide evidence that sperm is responsible for passing down certain traits,^[77] specifically, DNA methylation and acetylation patterns which were transmitted through at least three generations.^[77,78] Modifications to the epigenome in sperm have been seen to increase the frequency of disease in the future generations,^[78] as well as have the potential to influence behavior, such as greater risks for drug abuse and other addictive behaviors in offspring.^[79]

Detailing the mechanism of action behind transgenerational inheritance evident in these studies is challenged by the untraceable development of human germ cells *in vivo* that makes the isolating and studying of the limited number of PGC a difficult task.^[77] As mentioned previously, AFS cells may represent a suitable model for gamete precursors due to their similarities to PGCs, and therefore have the potential to provide an important resource for the study of human gamete formation, including epigenetic development. AFS cells in the modeling of human gametogenesis may play a pivotal role in the unveiling of the mechanisms responsible for transgenerational epigenetic inheritance.

Concluding Remarks

In addition to their projected use as a cell therapy, AFS cells exemplify an invaluable model for human genetic diseases, drug discovery, and potentially gamete formation. These are naturally occurring cells with a differentiation capacity greater than that of multipotent cells, and can be isolated without disrupting ethical parameters. With the advent of cell models well representative of human diseases, we can hope to reduce the need for animal models which are known to be unpredictable in their translation, and a costly endeavor.

This review aimed to highlight AFS cells as a novel model of human genetic diseases capable of filling the many gaps in knowledge presented in the literature. Importantly, these cells are easily obtained through amniocentesis, a commonly used procedure performed for prenatal diagnosis of genetic conditions. AFS cell models include diseases that are lethal in the course of pregnancy which, intuitively, is not possible of traditional dermal fibroblast derived iPS cell models. AFS cells avoid many of the limitations seen iPS cell models such as alterations due to aging, additional mutations, and epigenetic modifications that occur over a lifetime. Moreover, iPS cell generation can be a costly procedure that requires special facilities for manipulation with viral vectors, while AFS cells can be reprogrammed by viral and nonviral means. With the recent advent of CRISPR/Cas9 technology that enables genome editing with astounding precision, in theory, the number of disease models producible in AFS cells has dramatically increased. In sum, the unique characteristics of AFS cells attribute to their usefulness in a number of models that are beneficial for research in basic science through to clinical applications, enabling the pursuit of knowledge that may unobtainable through other means and models.

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Conflicts of interest

There are no conflicts of interest.

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