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Review

Amniotic Fluid-Derived Stem Cells: A Promising Resource for Cardiomyogenesis

Sajjad Sisakhtnezhad, Tahereh Merati

Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran

Abstract

Amniotic fluid stem cells (AFSCs) are one of the prenatal stem cell populations isolated from the amniotic fluid/membrane. They include different subpopulations from various sources of origin. AFSCs have important characteristics, including high self-renewal and proliferation capacity, immunocompatibility and anti-inflammatory properties, and differentiation potential into cell types of all three germ layers *in vitro*. Given the cardiomyogenesis potential of AFSCs and their importance in tissue engineering and regenerative medicine, this review aims to address the current findings regarding the characteristics of the AFSCs. Moreover, we present the strategies and methods used for the differentiation of AFSCs into cardiomyocytes *in vitro* and *in vivo*. This review also describes and discusses findings regarding the possible signaling pathways, the well-known molecular regulators, and modifications that are important for AFSCs and their differentiation potential into the cardiomyocytes. In general, this review indicates that AFSCs can efficiently differentiate into cardiomyocytes by different methods. Moreover, induction of the ERK signaling pathway, upregulation of epigenetic modifiers (GCN5, EZH2, SUZ12, DNMT1/2, and HP-1α) and the cell cycle regulators (p53, p21, Rb, and p130), suppression of HDAC1/2 and stemness markers (OCT4, NANOG, SOX2, KLF4, and REX1), the relative expression of miR-34a and miR-145, and induction of the expression of structural and functional-specific genes of cardiomyocytes (*GATA4, Nkx2.5, cTnT, MHC, Myh6, and Tnni3*) are the most important molecular changes during the differentiation of AFSCs into the cardiomyocytes.

Keywords: Amniotic fluid stem cells, cardiomyogenesis, characterization, epigenetics, mesenchymal stem cells, molecular mechanisms

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An Introduction to Amniotic Fluid Stem Cells

Amniotic fluid-derived stem cells (AFSCs) as a subpopulation of prenatal stem cells are attractive cells for stem cell-based research and therapeutic applications in regenerative medicine.[1, 2] Amniotic fluid (AF) is a protective, clear, slightly yellowish liquid that surrounds the fetus during pregnancy. AF is present from the formation of the gestational sac and accumulates in the sac of membranes known as the amnion. It is generally composed of water, chemical substances, and cells with morphologically heterogeneous characteristics.[2, 3] Amniotic fluid cells (AFCs) are categorized into three main subpopulations based on their morphological and growth characteristics: 1) Epitheloid (E-type) cells, which originate from fetal skin and urine; 2) Amniotic fluid specific (AF-type) cells, which derived from embryonic and extra-embryonic tissues during the process of fetal development and growth; 3) Fibroblastic (F-type) cells, which originating from fibrous connective tissue and dermal fibroblasts.[4, 5] Although amniotic fluid was first isolated and studied during the beginning of the 20th century,[6] the presence of progenitor cells in the AF was initially reported in 1993.[7] Interestingly, in 2004, Prusa and colleagues reported that AF has a population of *OCT4*-express-

Address for correspondence: Sajjad Sisakhtnezhad, PhD. Department of Biology, Faculty of Science, Razi University, Kermanshah, Iran
Phone: +988334274545 **E-mail:** s.sisakhtnezhad@razi.ac.ir; ssisakhtnezhad@gmail.com

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ing cells.[8] Subsequently, mesenchymal stem cells (MSCs) were derived from AF at different weeks of pregnancy.[9, 10] Finally, De Coppi introduced isolated AFSCs as cells that express both embryonic and adult markers.[11] Altogether, these findings indicate that amniotic fluid contains a heterogeneous subpopulation of fetal-originating stem/progenitor cells that also include AF-derived mesenchymal stem cells (AFMSCs).

AFSCs exhibit morphologies, phenotypic characteristics, and trilineage mesoderm (osteoblasts, adipocytes, and chondrocytes) differentiation potentials similar to MSCs. Different studies have also shown that AFSCs are positive for the expression of cell surface molecules including CD105, CD73, and CD90, and negative for CD45, CD34, CD133, CD14, CD11b, CD79α, CD19, and HLA-DR. Although AFSCs are known as multipotent cells, they are also reported as pluripotent cells in some studies.[11-13] Analysis and comparison of gene expression profiles of multiple clones of AFSCs revealed that they are positive for major histocompatibility (MHC) class I molecules (HLA-ABC), but weakly positive for MHC class II. They also express specific embryonic antigens, such as SSEA-4 and CD90 that are specific markers in the ESCs.[13] Moreover, nearly 90% of AFSCs express the specific factors of the embryonal carcinoma cells (ECCs), embryonic germ cells (EGCs), and embryonic stem cells (ESCs), like OCT4 and TERT.[9, 11, 14-16] Interestingly, the cultured AFSCs never show aging features and tumorigenicity behavior, even after long-period cultivation for more than two years *in vitro*.[11, 17-21] Molecular imaging techniques, which allow researchers to track transplanted pluripotent cells *in vivo*,[22] have demonstrated that unlike ESCs and induced pluripotent cells (iPSCs) that induce teratomas formation after injection *in vivo*, AFSCs have no tumorigenic potential after transplantation in mice.[23] This may result from genomic stability and epigenetic fidelity, which might be associated with the high expression of tumor suppressor *p53* by AFSCs. Tumor protein 53 is known as cell guard, because it controls cell proliferation as well as can induce some DNA repair systems and intrinsic apoptotic pathways.[24] Cozene et al. showed that *p53* is primarily expressed in the AFSC nuclei, where it maintains genomic stability and is an essential regulator of stem cell fate.[25]

Although Coppi et al. indicated that the differentiation ability of AFSCs lies between multipotent and pluripotent stem cells,[11] it recently reported that AFSCs have the potential to differentiate into three germ layers and specific lineage tissues *in vitro*.[13] Therefore, the results of different studies indicate that AFSCs can be classified as a new class of highly stable stem cells with intermediate plasticity properties between embryonic and adult stem cell types, and thus this makes them attractive cells for stem cell-based therapeutic applications in regenerative medicine. Interestingly, it has been shown that cell source, donor age, plating density, passage number, plastic surface quality, cell culture media and supplements (such as growth factors (GFs), chemokines, and cytokines), mechanical and electrical stimuli, and hypoxia affect characteristics and functions of MSCs *in vitro*.[26] Although Kunisaki et al. indicated that the antigenic expression profile of AFMSCs is not affected by gestational age or the type of media used for cell culture,[27] other studies reported that the phenotypic characteristics of AFSCs varied based on gestational age.[28-31] Therefore, according to these findings, we propose that the fetal stage from which amniotic fluid is collected and culture conditions may influence the potency of AFSCs *in vitro*. However, future studies are needed to confirm the effect of the source of AF and culture conditions on the characteristics and functions of AFSCs *in vitro*.

AFMSCs, as a subpopulation of AF-derived cells, have important characteristics. They have high self-renewal and proliferation capacity and express embryonic pluripotency and cell lineage biomarkers. Moreover, they have immunocompatibility and anti-inflammatory properties as well as can differentiate into different cell types of all three germ layers such as adipogenic, osteogenic, chondrogenic, myogenic, cardiomyogenic, endothelial, neuronal, and hepatic lineages.[32-34] Therefore, these potential characteristics make AFMSCs an attractive candidate for different applications such as disease modeling, drug screening,[35, 36] reprogramming, developmental and differentiation studies, tissue engineering, and regenerative medicine for human medical conditions including the treatment of neonatal diseases.[13, 35-39]

AFSCs can be differentiated into various cell types *in vitro* and *in vivo*. In this regard, cardiomyocytes are artificially derived from AFSCs *in vitro* by inducing a forced expression of cardiac-specific genes using different methods and protocols.[1, 20, 40-47] The AFSCs-derived cardiomyocytes may be used for different applications, including the treatment of human neonatal and postnatal heart diseases. A newborn infant's heart has an infinite potential for stem cell therapy and neonates might be the best candidates for stem cell therapy.[48] Therefore, given the cardiomyogenesis potential of AFSCs and their importance in stem cell-based therapy and regenerative medicine, in this review, we attempt to present and discuss the current findings regarding the characteristics of the AFSCs. In addition, herein we present the strategies and methods used for the differentiation of AFSCs into cardiomyocytes *in vitro* and *in vivo*. Furthermore, this review addresses and discusses findings regarding the possible signaling pathways, the well-known molecular regulators and modifications, especially epigenetic changes and their regulators and external influential factors, which are crucial for the AFSCs and their differentiation potential into cardiomyocytes.

Amniotic Fluid Mesenchymal Stem Cells

Nowadays, MSCs can be isolated from different fetal and adult tissues. Interestingly, the tissue source of MSCs can affect the outcome of cells and their characteristics *in vitro*. It has been found that the frequency of MSCs derived from bone marrow (BM) and adipose tissues (AD) is higher than other sources. In addition, it has been reported that AD-derived MSCs (ADMSCs) produce more copy numbers of MSCs *in vitro*.[26, 49, 50] Researchers have also introduced the amniotic fluid as an attractive and valuable source for the derivation of MSCs.[31, 51-53] Human AFMSCs (hAFMSCs) can be isolated from small volumes (2-5 ml) of second and third-trimester amniotic fluid and purified with three different methods[28, 54-56] (Fig. 1). Unlike the other AFCs, which are non-adhering cells,

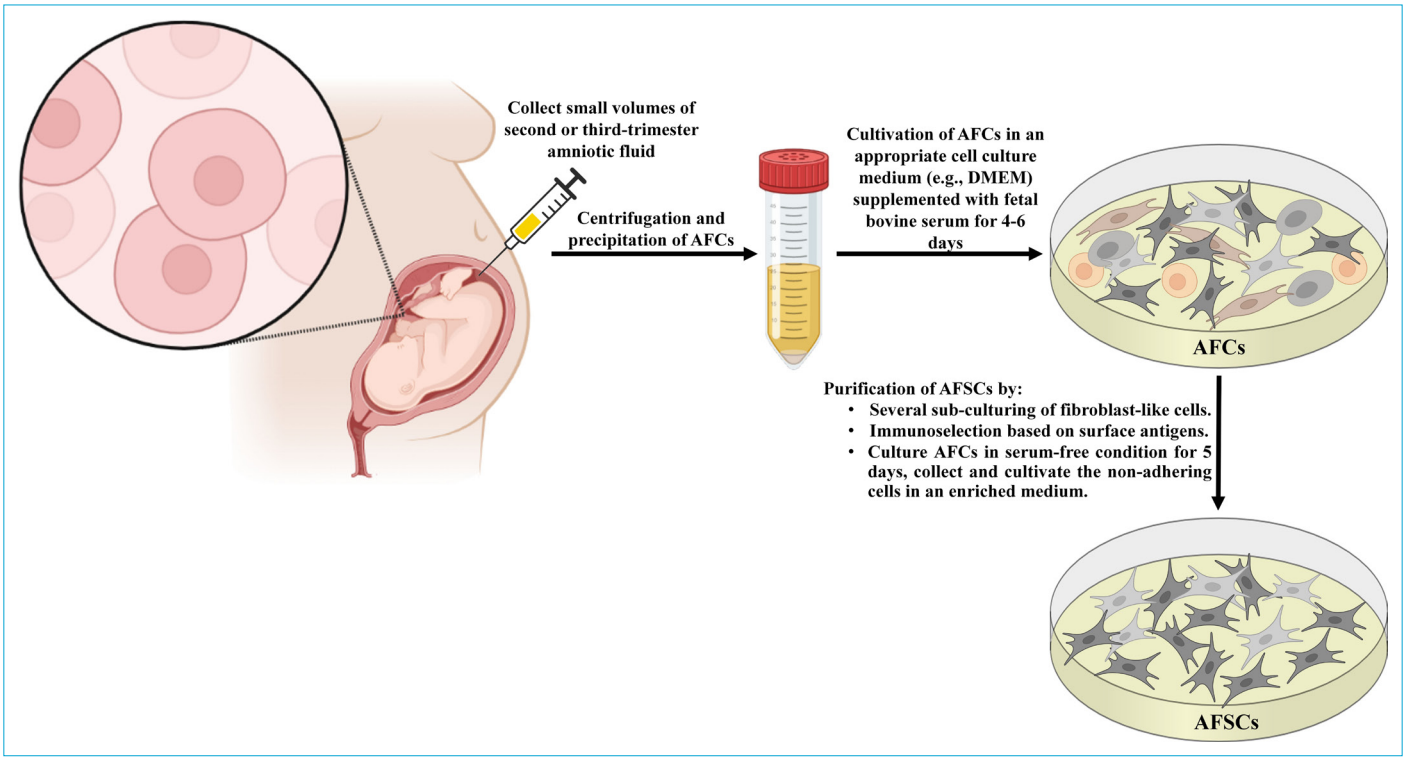

Figure 1. Schematic representation for the collection of amniotic fluid from a pregnant mother and the isolation and purification of AFSCs *in vitro*.

AFMSCs exhibit plastic adherent characteristics under normal culture conditions without feeder layers and have a fibroblast-like morphology as well as colony-forming ability *in vitro*. Indeed, AF-derived MSCs have a sharp slope proliferation and a remarkable ability to create a subculture that makes them potent for colony formation *in vitro*.[57] Antonucci et al. reported that AFMSCs have high self-renewal potential and can be expanded for more than 250 doublings without loss of chromosomal telomere length.[16] Therefore, AFMSCs are valuable cells, which can easily be derived from small volumes of AF. Moreover, they can stably be expanded *in vitro*.

Although the frequency of AF-derived MSCs is estimated between 0.9-1.5%, it has been reported that 2.7×10^5 cells can be approximately obtained from a primitive culture of AF.[58] However, it has also been demonstrated that the number of AFSCs cells increases with gestational age. In addition, pathological conditions can influence the number of AFSCs. For example, urogenital atresia and spina bifida increases and decreases the number of AF-derived stem cells, respectively.[59] Moreover, about 7.7×10^{23} AFMSCs were obtained after 27 passages and 66 cell doublings.[28] In another study, 18×10^6 AFSCs were obtained after four weeks of culture.[60] Furthermore, it found that AFMSCs survived and proliferated for 8 months in cultures,[28] therefore, they can be introduced as highly proliferative cells with a stable karyotype *in vitro*.

MSCs derived from various sources have different characteristics. There are significant differences between the differentiation potency of MSCs from different sources, which affects their usage for therapeutic applications in tissue engineering and regenerative medicine. For example, although menstrual blood-derived MSCs are superior in terms of immune inflammation, stress response, and neural differentiation potentials, they have weaker osteogenic and chondrogenic potentials, compared with umbilical cord-derived MSCs and bone marrow-derived MSCs (BMMSCs).[61] In addition, Ramkisoensing et al. showed that human embryonic stem cell-derived MSCs and fetal-derived MSCs such as AFMSCs, but not the MSCs with adult origin, can differentiate into three cardiac lineages, including cardiomyocytes, endothelial cells, and smooth muscle cells. Interestingly, they also found that the ability of MSCs to undergo functional cardiomyogenic differentiation is determined by the microenvironment of the cells, especially their communication with adjacent cell types by gap junctions. Moreover, they indicated that the gap junction protein Connexin 43 (CX43) may play an important role in the cardiomyogenic differentiation process.[62]

Nowadays, AFMSCs can be used in tissue engineering and regenerative medicine, in particular for the treatment of cardiovascular diseases;[63-65] because in addition to their high self-renewal potential and stability in long-term cell cultures,[11, 17-21] they also have multi-lineage differentiation capacity.[62] In this regard, Jiang and Zhang successfully isolated and cultured MSCs from AF using the direct adherence method. They reported the expression of *OCT4* by AFMSCs and confirmed that these cells are capable of multipotent differentiation. Furthermore, the tumorigenicity experiments carried out by Jiang and Zhang showed that AFMSCs are not tumorigenic. In addition, they showed that 5-azacytidine (5Aza) or transforming growth factor beta 1 (TGFβ1) treatments can convert AFMSCs into cardiomyocyte-like cells *in vitro*. How-

ever, they demonstrated that following combined treatment with 5Aza and TGFβ1, AFMSCs exhibited positive expression of GATA4, cTNT, and CX43, and a myofilament-like structure. Therefore, this study provides an efficient and practical method for the directional differentiation of AFMSCs, increases the effectiveness of the transformation of cardiomyocyte-like cells *in vitro*, and presents a promising strategy for the regeneration of myocardial cells.[63] Moreover, different studies have shown that AFMSCs promote wound regeneration through paracrine and immunomodulatory effects, epidermal differentiation, vascular and skin regeneration, and peripheral nerve injury repair.[65] Therefore, these findings indicate that AFMSCs are a promising candidate that can be used in tissue engineering and regenerative medicine, especially for the treatment of cardiovascular diseases.

Phenotypic Characteristics of AFMSCs

Phenotypic characterization of cells is critical for the identification, quantification, and isolation of a specific cell group in the mixed population using flow-cytometry. Phenotypic characterization of cells can be accomplished by staining them simultaneously with one or more antibodies that detect markers or antigens on the cells. Phenotypic characterization assays revealed that AFMSCs have gene expression profile characteristics that make them more similar to undifferentiated cells.[16] However, some modifications were detected in the phenotypic expression of AFMSCs when increased their passage number.[11, 28]

As previously mentioned, a study by Kunisaki et al. reported that the antigenic expression profile of AFMSCs is not affected by gestational age or the type of media used for cell culture. They showed that AFMSCs at any passage were positive for CD73 (SH3), CD105 (SH2), CD44, CD29, CD90, CD13, CD10, CD71, and HLA-A/B/C, and were negative for CD45, CD34, CD14, CD19, CD8, CD56, and CD31. In addition, they reported that there were no differences in antigen expression of AFMSCs based on gestational age or the type of culture medium used.[27] Nonetheless, other studies have shown that the antigenic expression profile of AFSCs is varied based on gestational age.[28-31] For example, Pipino et al. collected the amniotic fluid from 12 donors at different gestational ages. They observed the prevalence of the epithelial-like phenotype in 5, whereas the fibroblast-like morphology was predominant in 7 samples. They also demonstrated that epithelial- and fibroblast-like phenotypes showed slight differences in membrane markers, with higher CD90 and lower Sox2 and SSEA-4 expression in fibroblast-like than in epithelial-like cells; whereas CD326 was expressed only in the epithelial-like phenotype. The proteomic analysis also cleared that samples with a predominant epithelial-like phenotype showed a different profile than those with a predominant fibroblast-like phenotype. Furthermore, they did not show any significant differences in the differentiation potential of AFMSCs into osteoblasts, adipocytes, and chondrocytes.[29]

In addition, a study by Di Trapani revealed that first-trimester-derived AFSCs present some peculiar mechanisms of immune regulation that could be related to an early embryonic stage, while second- and third-trimester-derived AFSCs share many of the adult MSC immunomodulatory characteristics.[30] In another study, Savickiene and his colleagues characterized the derived AFMSCs from the second- and third-trimester of gestation. They found that AFMSCs derived from the late second- and third-trimester of gestation displayed similar MSC characteristics related to morphology, proliferation capacity, expression of specific cell surface and pluripotency markers, and multilineage differentiation potential. They also identified proteomic profiles of cultured AFMSCs from the late trimester of gestation and differentiated toward four distinct lineages. Moreover, the detailed comparative proteomic analysis of 250 proteins selected from more than 1400 proteins led to clarifying the differences in the expression of specific proteins in AFMSCs derived from different gestational ages.[31] Despite contradictory reports regarding the effect of gestational age and culture conditions on AFSCs characteristics, the antigenic expression profile that has been identified and reported for AFMSCs so far is presented in Table 1, in comparison with BMMSCs and ADMSCs.

Interestingly, the flow-cytometry assay carried out by Stefanidis et al. demonstrated the presence of *DAZL* and *c-Kit* expressing cells in the AFMSCs populations.[68] DAZL is an RNA-binding protein that is crucial for vertebrate germ cell development.[82] Moreover, c-Kit, a receptor tyrosine kinase, is involved in intracellular signaling and plays a crucial role in the gametogenesis process in vertebrates.[83, 84] Therefore, as previously reported for amniotic membrane-derived stem cells,[85] we suggest that the expression of DAZL and c-Kit genes in AFMSCs may enable these cells to demonstrate the plasticity and versatility potential to reprogram and transdifferentiate into the embryonic germ cells and their progenies. Accordingly, AFMSCs as competent cells may efficiently be used to convert into germ cell lineages for cell-based therapy applications and thus the treatment of some human infertilities. Therefore, future studies will be needed to examine and prove this valuable potential of AFMSCs *in vitro* and *in vivo*.

It has also been reported in many studies that approximately 90% of AFMSCs express the embryonic pluripotency markers, including *Oct4* and *TERT* genes at the mRNA and protein levels.[8, 10, 27] However, the disappearance of *Oct4* expression was seen at the 20th passage of AFMSCs and also they reach senescence at the 27th passage.[28] Therefore, it can conclude that the onset of AFMSCs aging may be at passages more than 20th. In addition to the embryonic pluripotency markers, AFMSCs at passages 4-8 express cell surface antigens like SH2 (low positivity until passage 8), SH3, SH4, CD29, hyaluronan receptor (CD44), and HLA-ABC (MHC class I) and low positivity for CD90 and growth factor beta receptor endoglin transforming (CD105) and ES marker CD117. Conversely, CD10, CD11b, CD14, CD34, CD117, HLA-DR, DP, DQ (MHC class II), and EMA expression were not detected and thus they were introduced as negative markers of AFMSCs.[10, 73, 86] Moreover, the other characterization studies confirmed the expression of the other typical antigens of ECCs and ESCs, including TRA-1-60, SSEA-3, and SSEA-4 in both unselected and c-kit positive AFMSCs at the 17th passage.[11, 28] It has also been proven that AFMSCs at the 17th passage express collagen types I, II, III, IV, and XII, fibronectin,

Table 1. Positive and negative antigenic expression profile of MSCs derived from amniotic fluid, bone marrow, and adipocyte tissues

MSC	Markers	Expression	Reference
AFMSCs	DAZL, c-Kit, Oct4, TERT, CD13, CD29 (β1-integrin), CD31, CD44 (HCAM-1), CD49e (α5-integrin), CD54 (ICAM-1), HLA-ABC (HLA-I), CD73 (SH2/3/4), CD90 (Thy1), CD105 (SH2/Endoglin), CD106, CD117, CD166, CD271, TRA-1-60, SSEA-4, Collagen types I, II, III, IV, and XII, Fibronectin, ICAM-1, α-SMA, CK18, Desmin, Vimentin, vWF, FSP, OCT3/4, NGFr, Flk-1	Positive	[4, 42, 60, 66-68]
	CDla, CD3, CD10, CD11b, CD14 (LCA), CD34 (gp105-120), CD45 (LPS-R), CD49d (α4-integrin), CD50 (ICAM-3), CD117, CD133 (Prorninin-1), HLA-DR/DP/DQ (HLA-II), EMA, Stro-1, Pan-cytokeratin	Negative	
BMMSCs	CD9, CD10, CD13, CD29, CD44, CD44H, CD49b/d, CD54, CD58, CD59, CD62L, CD71, CD73, CD90, CD105, CD106, CD119, CD120a, CD120b, CD121a, CD124, CD126, CD127, CD140, CD140b, CD146, CD166, CD271, GD2, BS1, a-SM actin, Alkaline phosphate, β-tubulin, FGFR1-4, FGF-2, CCR2a, CCR8, LNGFR, Osteonectin, STRO-1, Fibronectine, HLA-ABC, CXCR4, Collagen I/III	Positive	[60, 66, 69-80
	CD1a, CD3, CD4, CD5, CD11a, CD14, CD15, CD18, CD25, CD31, CD33, CD34 CD38, CD45, CD56, CD26E, CD123, CD133, CD144, HLA-DR, EGFR-3, SSEA-4, Myo-D, VEGFR-2PPARy	Negative	
ADMSCs	CD29, CD 54, CD90, CD105, CD106, CD146, CD166, 3G5, Alkaline phosphate	Positive	[68, 81]
	CD1a, CD14, CD31, CD34, CD44, CD45, STRO-1	Negative	

CD44 (homing cell adhesion molecule, HCAM), CD54 (Intercellular cell adhesion molecule-1; ICAM-1), CD31 (Platelet/endothelial adhesion molecule-1, PECAM-1), CD106 (Vascular cell adhesion molecule-1; VCAM-1), alpha-smooth muscle actin (α-SMA), CK18, desmin, vimentin, vWF and FSP.[28] Taken together, the gene expression profile of AFMSCs indicates that these cells may be pluripotent stem cells that as well as less differentiated than BM- and AD-MSCs. In addition, although it has been reported that the AFSCs gene expression profile is almost not influenced by gestational age and culture conditions during expansion *in vitro*,[27] other studies have provided contradictory results.[28-31] Therefore, further studies still need to be carried out to examine the exact effects of gestational age and culture conditions on the characteristics and fate of AFMSCs.

Transdifferentiation of AFSCs into Cardiomyocytes

There are different studies regarding the ability of various stem cells to transdifferentiate into other cell types *in vitro* and *in vivo*. The results of these studies indicate that some of these cells are capable to differentiate into cardiomyocytes and some are not. For example, Murry et al. demonstrated that hematopoietic stem cells cannot transdifferentiate into cardiomyocytes.[87] In addition, Reinecke and colleagues showed that skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting.[88] However, a study by Iijima et al. indicated that beating is necessary for the transdifferentiation of skeletal muscle-derived cells into cardiomyocytes. They also found that treatment of nifedipine or culture in Ca^{2+}-free media suppressed the contraction of cardiomyocytes and inhibited skeletal muscle cells to express cardiac-specific proteins.[89] Moreover, nowadays it has been shown that differentiated cells such as fibroblasts,[90-92] pluripotent stem cells such as ESCs and iPSCs,[93, 94] and adult stem cells such as MSCs[95, 96] and spermatogonial stem cells[97] can transdifferentiate into cardiomyocytes by different methods, including ectopic expression of cardiomyogenic genes, co-culture system, and treatment with cardiomyogenic induction media and small-molecule chemicals.[89, 90, 96, 98]

As described before, AFSCs have the potential to differentiate into various cells *in vitro*.[32-34] Moreover, because AFSCs have some pluripotent features and also are less differentiated than the adult-derived MSCs, therefore, they are one of the most valuable cells that can be employed for unraveling the bases of differentiation, physiology, biochemistry, and potential pathologic processes during embryonic development and adult cell differentiation. Nowadays, it has been shown that AFSCs can efficiently be transdifferentiated to cardiomyocytes *in vitro* and *in vivo* by different methods and protocols (Table 2 and Fig. 2), therefore, they are promising cells for the treatment of some human cardiac failures, especially neonatal heart disorders. Herein, we present and discuss the most developed systems for the differentiation of AFSCs into cardiomyocytes *in vitro* and *in vivo* and also their efficiency for therapeutic applications.

Co-Culture System

About ten years ago, Yeh et al. differentiated human AFSCs (hAFSCs) into cardiomyocytes using a mimicking differentiation milieu. They showed that when AFSCs co-cultured with rat neonatal cardiomyocytes or in endothelial cell growth medium-2 (ECGM-2) enriched with vascular endothelial growth factor (VEGF), they dif-

Table 2. In vitro and in vivo differentiation models of AFSCs into cardiomyocytes

Differentiation method	System	Cardiomyogenic molecular biomarkers	Reference
Co-culture of human AFSCs (hAFSCs) with rat neonatal cardiomyocytes and transplantation of the AFSCs into the peri-infarct area of the immune-suppressed rats with induced myocardial infarction	*In vitro* and *in vivo*	NKX2.5, α-actinin, Cardiac troponin T, and CX43	[40]
Cultivation of AFSCs in ECGM-2 supplemented with VEGF	*In vitro* and *in vivo*	NKX2.5, α-actinin, Cardiac troponin T, and CX43	[40]
Systemic injection of allogenic rat AFSCs (rAFSCs) into adult rats with cardiac ischemia-reperfusion injury	*in vivo*	Thymosin beta-4	[41]
Co-culture of c-Kit-sorted and GFP-positive rAFSCs with neonatal rat cardiomyocytes	*In vitro*	Cardiac genes troponin I (cTnI) Sarcomeric α-actinin (cαA)	[42]
Treatment of hAFSCs with 5-aza-2'-deoxycytidine	*In vitro*	CX43	[20]
Intravascular injection of hAFSCs into rats	*In vitro*	Reduction in BNP and pro-inflammatory cytokines	[43]
Co-culture of hAFSCs with cardiomyocytes	*In vitro*	Natriuretic peptide, CD31, α-SMA	[1]
Reprogramming of hAFMSCs into iPSCs using non-integrating Sendai virus (SeV) expressing OCT4, SOX2, c-MYC, and KLF4, then exposure of these iPSCs to cardiogenic differentiation conditions using different supplements, growth factors, and signaling inhibitors	*In vitro*	MYH6, MYL7, TNNT2, TTN, and HCN4	[44]
Treatment of hAFMSCs with DNA methyltransferase (DNMT) inhibitors decitabine, zebularine, RG108 alone or combined with zebularine and p53 inhibitor pifithrin-α	*In vitro*	NKX2.5, TNNT2, MYH6, DES, and CX43	[45]
Treatment of hAFMSCs with angiotensin II, retinoic acid, epigallocatechin-3-gallate (EGCG), and vitamin C	*In vitro*	NKX2.5, MYH6, TNNT2, DES, CX43 and NKX2.5	[46]
Treatment of hAFMSCs by human platelet lysate and 5-azacytidine	*In vitro*	GATA4, cTNT, CX43 and NKX2.5	[47]

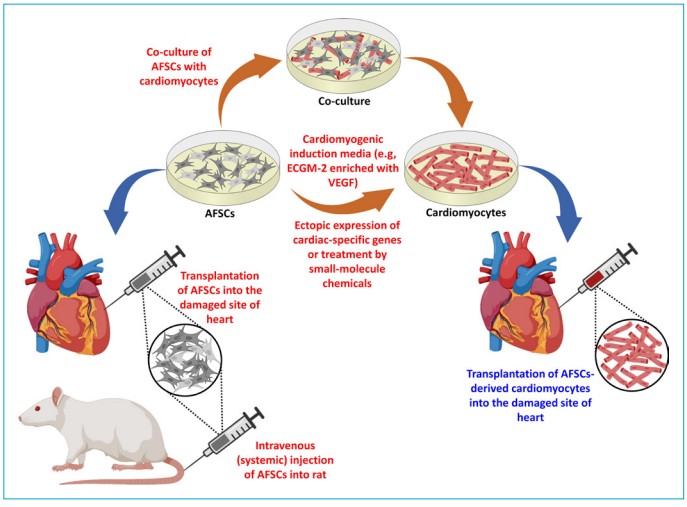

Figure 2. Different methods for the differentiation of AFSCs into cardiomyocytes *in vitro*.

ferentiated into cardiomyocyte-like and endothelial cell lineages, respectively. They also examined the therapeutic potential of the hAFSCs transplanted with a needle into the peri-infarct area of the immune-suppressed rats with induced myocardial infarction. Interestingly, the attenuation of left ventricle remodeling and thus a decrease in the infarcted tissues with the observation of high vascular density after 4 weeks confirmed that the hAFSCs can stimulate cardiomyogenesis and angiogenesis *in vivo*.

At the molecular level, the expression of the cardiac-phenotype antigens such as Nirenberg, Kim gene 2 homeobox 5 (NKX2.5), α-actinin, Cardiac troponin T, and CX43 (indicating an enhancement of cardiac and vascular cell connections) was also detected in hAFSCs-derived cardiomyocyte-like cells in this study [40]. Therefore, this study suggests that hAFSCs are potent cells to differentiate into cardiomyogenic and endothelial lineages for the repair of cardiac disorders.

In another study, co-culture of hAFSCs with rat cardiomyocytes or cultivation of them in the cardiomyogenic induction media induced the expression of proteins specific for cardiomyogenesis such as atrial natriuretic peptide and alpha-myosin heavy chain (α-SAM)), endothelial (CD31 and CD144), and smooth muscle cells (alpha-smooth muscle actin) in the differentiated cells.[1] However, although these studies indicated that hAFSCs can differentiate to cardiovascular lineages *in vitro* by co-culture with cardiomyocytes from other species like rats, their clinical application for cell therapy of myocardial infarction may hamper by their phenotypic instability, viral transmission from animals to humans, and ethical problems. Therefore, more studies will be needed to investigate these issues.

Collectively, the *in vitro* co-culture system of cells is closer to the tissue microenvironment, because it provides a more realistic environment that mimics the phenotypic and functional characteristics of the animal body. Therefore, this system has the advantage that can be used as a good platform to study the interaction

between different cell types and their impacts on phenotypic and functional characteristics of each other *in vitro*. The co-culture system is also a simple and attractive technique in cell biology studies and applications, especially for understanding cell-cell interactions and communications during development and differentiation processes.[99-101] During the past decade, as indicated above, the co-culture of AFSCs with cardiomyocytes provides a good platform, which is not technically challenging, to produce AFSCs-derived cardiomyocytes for different applications. Even though the co-culture system is efficient for the AFSCs differentiation into cardiomyocytes, it may be affected by cell type, species, and culture conditions, especially the type of *in vitro* culture (2D or 3D) and the interaction between cells. For example, the differentiation of rat bone marrow-derived MSCs into cardiomyocytes is achieved by direct co-culture with neonatal cardiomyocytes but not adult cardiomyocytes.[102] Therefore, more investigation is required to be done to determine the influences of external influential factors on the AFSCs differentiation into cardiomyocytes *in vitro* and their efficient clinical applications in the human body.

Systemic Injection or Direct Transplantation of AFSCs

Systemic injection or direct transplantation of AFSCs into the heart is also crucial methods for transdifferentiation of them into cardiomyocytes *in vivo*. In this regard, Bollini et al. confirmed the paracrine therapeutic effects of the intravascular transplantation of human xenogeneic AFSCs in a rat model of acute myocardial infarction.[41] They showed that the systemic injection of hAFSCs that secreted putative paracrine factors such as thymosin beta-4 (Tb-4), which is an actin monomer binding protein with cardioprotective properties,[103] improved myocardial cells survival and decreasing the infarct size from about 54 to 40 %.[41] They also successfully differentiated the c-Kit-sorted and GFP-positive rat AFSCs (rAFSCs) into cardiomyocytes by a co-culturing system with neonatal rat cardiomyocytes. They showed that rAFSCs have a propensity to acquire a cardiomyogenic phenotype *in vitro* and to preserve cardiac function in the heart of animals with ischemia/reperfusion injury, even if their potential may be limited by poor survival in an allogeneic setting.[42]

Furthermore, a study by Castellani et al. demonstrated that the intravascular injection of hAFSCs caused favorable effects on skeletal muscle remodeling in a well-established rat model of right heart failure. They detected a reduction in the natriuretic peptide (BNP, a surrogate factor for heart injury, and pro-inflammatory cytokines) in the hAFSCs, while they homed to the heart and lung. Therefore, they reported that hAFSCs may have beneficial applications for the treatment of heart failure due to pulmonary hypertension.[43] In general, these studies indicated that AFSCs can directly transplant into the damaged site of the heart or can intravenously inject into the body of an organism to migrate to the heart for cardiomyogenesis and thus the treatment of some heart failures. Although, these studies indicated that the direct transplantation of AFSCs or the intravenous injection of them are efficient methods to induce cardiomyogenesis *in vivo* for repair and regeneration of the cardiac muscular tissue, it may be affected by abnormal differentiation of AFSCs, out-of-control cell proliferation and thus tumorigenesis, and immunorejection. Therefore, further studies are required to investigate these issues regarding the application of AFSCs for cardiomyogenesis *in vivo* as an efficient and valuable technique to treat some heart failures in humans.

Treatment of AFSCs by Small-Molecule Chemicals

In addition to the co-culture of AFSCs with cardiomyocytes *in vitro* and transplantation or injection of them *in vivo*, it has also been found that small-molecule chemicals like 5-aza-2'-deoxycytidine,[20] the cocktail of compounds including hyaluronic, butyric, and retinoic acids,[104] and the mixture of human platelet lysate and 5-azacytidine[47] can be used to differentiate the AFSCs into cardiomyocytes. Guan et al. demonstrated the potential of 5-aza-2'-deoxycytidine, as an epigenetic modifier drug, for the differentiation of hAFSCs into cardiomyocytes. They found that 5-aza-2'-deoxycytidine-treated hAFSCs converted into cardiomyocytes that expressed CX43, as a marker of cardiomyogenic differentiation.[20] Thus, it concludes that the hAFSCs can differentiate into cardiomyocyte-like cells through epigenetic modulators like 5-aza-2'-deoxycytidine. Furthermore, Maioli et al. showed that the mixture of hyaluronic, butyric, and retinoic acids efficiently induces cardiomyogenesis in hAFSCs *in vitro*.[104] They reported that these three chemicals significantly downregulated the expression of genes controlling pluripotency and plasticity of stem cells (*Sox2, Nanog, and Oct4*) in hAFSCs However, at this point, the expression of genes controlling the appearance of cardiogenic and vascular lineages upregulated in the treated cells. Therefore, these findings reveal that the conversion of AFSCs into differentiated progeny is mechanistically achieved by long-lasting changes in the gene expression of the cells.[105, 106]

Overall, until now various small-molecule chemicals have been identified that can stimulate the cardiomyogenesis process in AFSCs. The most significant advantages of small-molecule chemicals for the induction of cardiomyogenesis in AFSCs are that they are easy to manufacture, store, and administrate. The use of small-molecule chemicals is also cost-effective for the induction of reprogramming and differentiation processes, compared with gene manipulation and transcription factor-mediated protocols. In addition, their biomedical effects are dose-dependent, rapid, and specific, therefore, allowing for very precise temporal and functional control *in vitro* and *in vivo*. Moreover, these small-molecule chemicals aid the progression to co-culture-free protocols of AFSCs cultivation and differentiation, which accelerate the differentiation methods. They also reduce the use of more expensive recombinant products. Despite these advantages, due to probable toxicity or other dangerous side effects such as off-target action, the identification of highly active and specific small molecules that be suitable for clinical applications is a difficult task.

Factors Influencing the Cell Cycle, Stemness, and Epigenetics of AFSCS

A lot of molecular studies have revealed that the self-renewal and differentiation potential of embryonic stem cells and their progenitor cells are controlled through gene expression regulatory

mechanisms that are mediated by dynamic epigenetics regulation.[107] Epigenetic modification is a reversible and heritable process that is conducted to the modifications in gene expression without changes in the genomic sequences, while attributing to chromatin alteration or packaging that affects the accessibility of DNA for transcription modulators.[108] The major epigenetic mechanisms include DNA methylation, chemical modifications of histone protein tails such as acetylation, methylation, phosphorylation, and ubiquitination, and small and long non-coding RNAs-mediated regulatory events.[109] Epigenetic modifications through inducing chromatin remodeling events control the heritable cellular memory of gene expression and thus influence the characteristics and functions of cells. DNA and histone methylation is associated with DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs), respectively. DNMTs catalyze DNA methylation at cytosine positions within CpG islands and HMTs catalyze methylation on lysine and arginine amino acid residues of the amino terminus of histone proteins.[110] DNA methylation causes gene silencing, whereas depending on the type of histone and the amino acid residues that changed, histone modifications lead to either gene activation or repression. Histone acetylation is also a well-known epigenetic modification. The converse functions of histone acetyltransferases (HATs) and histone deacetylases (HDACs) are responsible for the acetylation and deacetylation of histones on the lysine residues. In general, acetylation and deacetylation of histone proteins correlated with the activation and repression of genes, respectively. The common histone acetylation marks in the transcriptionally active genes are H3K9ac and H4K16ac.[111]. In addition to DNA methylation and histone modifications, non-coding RNAs such as microRNAs also have a leading role in the epigenetic machinery.[112]

AFSCs are highly proliferative, pluri- or multi-potent, and immunocompatible cells that homing to damage and tumor sites in response to inflammatory factors.[28, 57, 113-115] Epigenetic modifications through inducing chromatin remodeling regulate the expression of genes associated with self-renewal, differentiation, migration, and immunomodulatory potentials of AFSCs. The recognition of epigenetic events, their influencing factors, and the complexity of the interaction between them in AFSCs are critical for understanding the molecular basis under the potential characteristics of these cells. Moreover, this valuable information has to be taken into account for the development of protocols to improve the potential of AFSCs for different applications in tissue engineering and regenerative medicine. Therefore, here we review the recent findings related to factors influencing the cell cycle, stemness, and epigenetics of AFSCs.

Savickienė et al. have shown that MSCs derived from amniotic fluid of normal gestation and with fetus abnormalities demonstrated diversity in their proliferation and senescence. They also found that the senescence process of AFMSCs during culture expansion and passaging is associated with the alterations in the expression of genes regulating cell cycle (p16, p21, p53, and ATM), stemness transcription factors (Oct4, Nanog, Sox2, and Rex1), miRNAs (mir-17 and mir-21), and epigenetic regulators or chromatin modifiers including DNMT-1, histone deacetylase-1 (HDAC-1) and polycomb group proteins (PGCs) such as enhancer of zeste homolog 2 (EZH2), suppressor of zeste 12 (SUZ12), and BMI1, which induce the production of repressive histone marks (H3K9me3 and H3K27me3).[116] Moreover, in another study Savickienė and colleagues reported that histone modifications pattern associated with a state of MSC cultures derived from AF of normal and fetus-affected gestations. Comparison of AFMSCs, which derived from normal gestation and donors with genetic or multifactorial fetal diseases, displayed distinct growth and immunophenotype characteristics associated with the alterations in global DNA methylation, the pattern of acetylated histones H3 and H4, and dysregulation of both methylated histones H3K27 and H3K9.[117] These findings indicate that epigenetic factors, which are crucial for the AFMSCs senescence process, may affect by the donor individuality or fetus malignancy status. Therefore, it can conclude that senescence-associated molecular and epigenetic changes during AFMSCs cultivation are related to the extent of MSCs characteristics that have to be taken into account for their therapeutic application. However, Zentelytė et al. study indicated that no significant differences could be found in the expression levels of all epigenetic or pluripotency markers between undifferentiated AFMSCs derived from normal and fetus-damaged gestations and between their differentiation potential into mesodermal lineages, including adipocytes or osteoblasts.[118] Therefore, further studies still need to be carried out to investigate epigenetic, stemness, and differentiation markers and their regulators in AFMSCs derived from normal and fetus-affected gestations.

In addition to the source of AFSCs and their number of passages, chemical factors can also affect the cell cycle, stemness, and epigenetics of AFSCs. In a recent study, the epigenetic effects of the cancer treatment chemotherapy drugs, including Cisplatin, Bleomycin, and Etoposide (CBE), were examined on stemness, proliferation, apoptosis, and chemosensitivity of hAFSCs by evaluating the global DNA methylation and the gene-specific DNA methylation for the imprinted gene *H19* and also some microRNAs.[119] It showed CBE is cytotoxic for hAFSCs in a time and dose-dependent manner. In addition, hyper-methylation for Bleomycin and Etoposide exposure and hypo-methylation for Cisplatin exposure was observed in the hAFSCs genome. They also revealed that although Cisplatin exposure significantly decreased the global 5'-methylcytosine (5'-mC) percentage of the genome, Bleomycin and Etoposide exposure significantly increased 5'-mC levels in DNA. Furthermore, it found that CBE influenced the expression of protein-coding genes associated with the pluripotency and germline lineage and the expression of miRNAs involved in the self-renewal, apoptosis, and chemosensitivity of hAFSCs. In this regard, Cisplatin and Etoposide mediated an inhibitory effect on the expression of pluripotency genes (*Oct4, SOX2, KLF4, c-MYC, and NANOG*), concomitant with the downregulation of germline markers (*Stella, Fragilis, VASA, DAZL, STRA8, PIWIL2, BOLL, and SYCP3*), particularly meiotic stage markers (*BOLL and SYCP3*). On the contrary, Bleomycin induced the activation of core pluripotency genes (*Oct4, NANOG, and SOX2*) and the most of premeiotic and all meiotic markers at the transcriptional level. Moreover, it reported that Bleomycin and Etoposide by upregulation

of hsa-miR-106b-5p, miR-185-5p, let-7a-5p and downregulation of miR-17-3p influenced the hAFSCs pluripotency and proliferation. Although Cisplatin had no significant effects on the expression of these miRNAs, it and Bleomycin induced apoptosis via upregulation of miR-34c-5p in hAFSCs by targeting p53. It was also declared that CBE induced apoptosis and chemosensitivity in hAFSCS through upregulation of miR-34c-5p and miR-449a, respectively.

Increasing evidence suggests that *p53* triggers various biological processes in differentiated somatic and also stem cells. The results of different studies provide insight into the profound roles of *p53* that govern the various potentials of stem cells to enable proper development, tissue regeneration, and heart injury-free life. Phermthai et al. examined *p53* mutation and epigenetic imprinted insulin growth factor 2 *(IGF2)/H19* gene analysis in MSCs derived from amniotic fluid, amnion, endometrium, and Wharton's jelly. Interestingly, they found that AFMSCs sustained stable *p53* expression levels throughout long periods of culture and showed a high level of *IGF2* with a stable pattern and level of *H19*, as compared with MSCs derived from other tissues.[120] Therefore, it concludes that p53-mediated high genomic stability and epigenetic fidelity make AFMSC a safe candidate for stem cell-based therapeutic applications. In addition, investigating the expression of this gene in AFSCs derived from normal and fetus-affected gestations and in the cells cultured in different conditions can be important for the effective use of these cells in therapeutic applications.

In general, the findings of these studies suggest that the epimiRNAs control of the gene expression levels in AFSCs is a critical mechanism to maintain the homeostasis of the cell characteristics. In this regard, the epigenetic regulators, including DNMT-1, HDAC-1, EZH2, SUZ12, and BMI1, which influence the global 5'-mC, H3K9me3, and H3K27me3 rate, stemness markers such as OCT4, SOX2, NANOG, KLF4, c-MYC, and REX, miRNAs like let-7a-5p, mir-17, miR-17-3p, mir-21, miR-34c-5p, miR-106b-5p, miR-185-5p, and miR-449a and the cell cycle regulating proteins such as p53, p16, p21, and ATM are the most well-known factors that affecting the AFSCs characteristics. It has also been found that epigenetic and stemness markers and their regulators in AFSCs can be affected by external influential factors such as gestation-related conditions, culture conditions, and chemical drugs. Therefore, more investigations should still be conducted to determine the exact effects of external factors influencing epigenetic and stemness markers and their regulators in AFSCs to expedite the progress of stem cell-based therapeutics in regenerative medicine.

Epigenetic Regulation During the Differentiation of AFMSCS into Cardiomyocytes

Developing a well-defined and efficient cardiomyogenic protocol is critical for producing suitable cardiomyocytes for clinical applications. So far, different methods and protocols have been employed to differentiate MSCs isolated from different sources into cardiomyocytes.[121] However, it has been shown that combination treatment with cytokines and growth factors, including bone morphogenetic protein (BMP), insulin growth factor (IGF), and fibroblast growth factor (FGF) and the activation of their signaling pathways (Fig. 3), is a potentially effective and reliable treatment

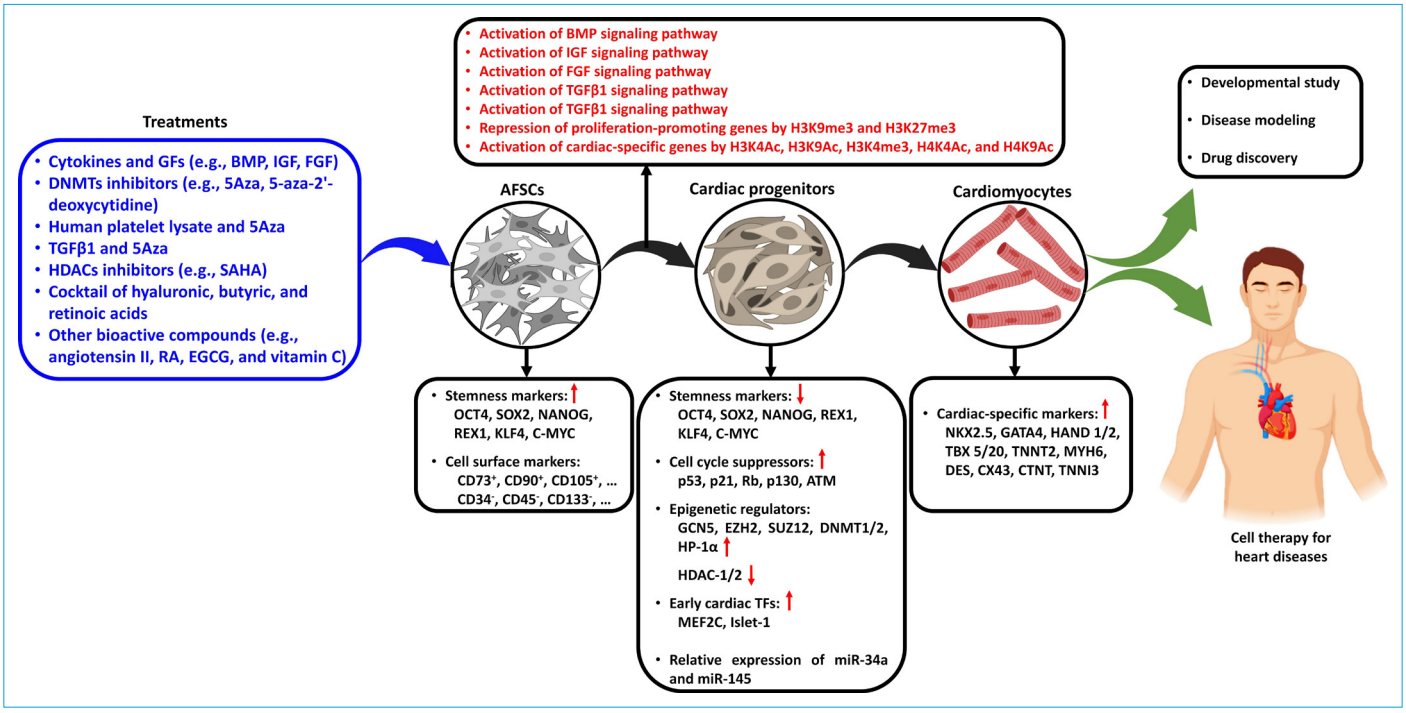

Figure 3. The most well-known external influential factors and the induced signaling pathways and the molecular changes and mechanisms during the differentiation of AFSCs into cardiomyocytes *in vitro*.

modality for the differentiation of MSCs into cardiomyocytes.[121, 122] Under combination treatment with these cytokines and GFs, MSCs commit to the cardiac lineage. The produced cardiac progenitors are proliferative, express significantly high levels of early cardiac transcription factors (TFs) such as insulin gene enhancer-binding protein ISL-1 (Islet-1) and myocyte-specific enhancer factor 2C (MEF2C) and possibly low levels of OCT4/SOX2. Continued induction will activate the expression of cardiomyocyte-specific transcription factors such as NKX2.5, GATA4, HAND 1/2, and TBX 5/20 in the progenitor cells and convert them into early cardiomyocytes. These cells express sarcomeric and structural proteins and demonstrate functional activity synonymous with mature contracting cardiomyocytes.[121]

The specific transformative steps from MSCs to the mature cardiomyocyte state are determined in large part by changes in gene expression (Fig. 3). Nowadays, epigenetic modifications have been accepted as one of the essential phenomena behind changes in gene expression during cardiac degeneration and regeneration, but the mechanisms underlying these processes are not well understood. Increasing evidence indicates that epigenetic changes, including DNA methylation and histone acetylation, are important modifications during the differentiation of MSCs into cardiomyocytes.[123] For example, it has been investigated that insulin gene enhancer-binding protein ISL-1 (Islet-1) through inducing histone acetylation of the lysine residues at the amino terminus of the chromatin core histones and DNA methylation stimulated MSCs to differentiate into cardiomyocyte like cells. It has been found that Islet 1 caused upregulation of the general control of amino acid biosynthesis protein 5 (GCN5) and mediated the binding of GCN5 to the promoters of GATA binding protein 4 (GATA4) and NK2 homeobox 5 (NKX2.5). Islet-1 also downregulated DNMT-1 expression and diminished its binding to the *Gata4* promoter.[124]

Evidence has been accumulating during the past decade indicating that AFMSCs could occupy a niche in the stem cell hierarchy the same as pluri- or multi-potent stem cells with the potency to differentiate into three germ layers (mesoderm, ectoderm, and endoderm) cells, especially cardiomyocytes.[125] Epigenetic events have critical roles in the AFMSCs fate determination, especially their differentiation into cardiomyocytes. A study by Gasiunienė et al. reported that DNMTs inhibitors, including decitabine, zebularine, RG108 alone and in combination with zebularine and pifithrin-α (*p53* inhibitor), lead to cardiomyogenic differentiation of AFMSCs. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis revealed that all agents could change the cell phenotype by upregulation of the relative expression of the main cardiac genes *Nkx2.5, Tnnty2, Myh6, and Des* as well as the cardiac sodium, calcium, and potassium ion channels genes. Additionally, western blot and immunofluorescence information detected an increase in CX43 levels. In addition to cellular energetic and mitochondrial function analysis using the Seahorse analyzer, these results also indicated metabolic transdifferentiation of AFMSCs into cardiomyocyte-like cells. Moreover, the upregulation of the *p53* and *p21* genes and relative expression of miR-34a and miR-145 demonstrated the cell cycle regulation at the G0/G1 phase. Also, the evaluated levels of EZH2, SUZ12, DNMT1, HDAC1, HDAC2, and heterochromatin protein-1α (HP-1α), which are known as chromatin remodeling proteins, and the rate of activating histone modifications exhibited rearrangements of chromatin after the induction of cardiomyogenic differentiation.[45]

In addition, in a recent study by Markmee et al. human platelet lysate and 5-azacytidine were used to induce the differentiation of AFMSCs into cardiomyocyte-like cells.[47] Results indicated higher viability and upregulation of cardiomyogenic specific genes, including *Gata4*, Cardiac troponin T (*cTnT), Cx43, and Nkx2.5*, in the treated AFMSCs. Moreover, following combined treatment with 5Aza and TGFβ1, AFMSCs exhibited positive expression of cardiac-specific markers (GATA4, cTNT, and CX43), and a myofilament-like structure under transmission electron microscopy.[63] 5-azacytidine is a demethylating agent that is used as a cardiomyogenic inducing factor. Indeed, as a synthetic analog of cytosine, 5-azacytidine can epigenetically induce the expression of genes involved in cardiomyogenesis by suppressing their DNA methylation.[126] Mechanistically, the significant upregulation of phosphorylated cardiomyogenic specific genes, such as *cTnT and Nkx2.5*, is induced through the extracellular signal-regulated kinase (ERK) signaling pathway that leads to the AFMSCs differentiation into cardiomyocytes.[127, 128] Altogether, according to the literature review of the previous studies, we suggest that DNMTs inhibitors may through suppressing DNA methylation and also the activation of signaling pathways such as ERK, and thus the induction of cardiomyogenic-specific genes expression stimulates the cardiomyogenesis potential of AFMSCs. In addition, the identification and recruitment of new DNMTs inhibitors may help to develop new and efficient protocols for the differentiation of AFMSCs into cardiomyocytes.

It has also been proven that the prevention of histone deacetylation by suberoylanilide hydroxamic acid (SAHA) is much more crucial than DNA methylation through 5-azacytidine during cardiomyogenic differentiation.[129, 130] Previous studies demonstrated the importance of GCN5, which is the catalytic subunit of several related HAT complexes, for the histone 3 (H3) acetylation of the promoters of *Gata4 and Nkx2.5* genes in murine and rat MSCs and thus their cardiomyogenesis.[124, 131] However, there is no report regarding the role of GCN5 during the differentiation of AFSCs into cardiomyocytes. Therefore, we suggest that the GCN5 role should not be overlooked during cardiomyogenesis from AFSCs and further studies should be done to elucidate the possible function of this factor in this differentiation process. In addition, there is evidence suggesting a reduction in the *HDAC1* and HDAC2 protein levels associated with the increased expression of structural and functional-specific genes of cardiomyocytes.[132] In this regard, Wang and colleagues showed that the knockdown of *Hdac1 or Hdac2* in BMMSCs upregulated the expression of the cardiomyocyte-specific genes *Myh6 and Tnni3* via both H3 (histone 3) and H4 (histone 4) acetylation on their corresponding promoters.[133] In accordance with these findings, a previous study by Lu et al. also indicated that the *Hdac1* knockdown may promote the directed differentiation of BMMSCs into cardiomyo-

cytes through influence on the cardiac-specific genes, including *Gata4, Nkx2.5, cTnT*, and myosin heavy chain (MHC).[134] In general, these studies indicated that histone acetylation is a critical process during the differentiation of MSCs to cardiomyocytes. Therefore, scientists can develop new and efficient protocols via the identification of HDACs inhibitors for the differentiation of MSCs into cardiomyocytes.

Recently, in another study, Gasiunien et al. showed the potency of the biologically active compounds, including angiotensin II, retinoic acid (RA), epigallocatechin-3-gallate (EGCG), and vitamin C, on the AFMSCs differentiation into cardiomyocytes. They observed the expression of cardiac-specific genes, including *Nkx2.5, Myh6, Tnnt2, Des*, and cardiac sodium, calcium, and potassium ion channels genes in the AFMSCs-derived cardiomyocytes. In addition, they reported increased levels of the CX43 and NKX2.5 proteins in the differentiated cells. Moreover, the extracellular flux assay demonstrated enhanced oxidative phosphorylation for energy production. Additionally, altering in epigenetic markers associated with transcriptionally active (H3K4me3, H3K9Ac, and H4hyperAc) or repressed (H3K27me3) chromatin indicated that the investigated biomolecules epigenetically influence the AFMSCs to differentiate into cardiomyocytes progenitors *in vivo*.[46] In accordance with these findings, a previous study by Sdek et al. also reported that methylation and acetylation of H3 play a critical role in cardiac growth and differentiation. They found that actively proliferating embryonic cardiomyocytes enriched for H3K9Ac and H3K4Ac, express high levels of proliferation-promoting genes. Over time, however, histone acetylation (H3K9Ac and H3K4Ac) decreased, and H3K9me3 and H3K27me3 were predominated. In this regard, they also showed that terminally differentiated cardiomyocytes express high levels of retinoblastoma (Rb) and p130, which serve as a bridge to link H3K9me3 and heterochromatin formation through their interaction with HP-1γ.[135] Besides, Wang et al. demonstrated that at the promoter regions of cardiac-specific genes, including *Myh6, Myl2, Actc1, Tnni3, and Tnnt2*, H3 and H4 acetylation (as a mark indicative of gene activation) was higher in cardiac stem cells than MSCs.[133] Therefore, these studies suggest that epigenetics modifications such as H3K4Ac, H3K9Ac, H3K4me3, H4K4Ac, and H4K9Ac, which are associated with euchromatin formation, are critical for the activation of cardiac-specific genes expression. Moreover, the repressive histone marks H3K9me3 and H3K27me3, which mediated heterochromatin formation, stably repress the expression of proliferation-promoting genes and maintain the terminally differentiated phenotype of cardiomyocytes. Although it demonstrated that epigenetic modifications are critical for heritable changes in gene expression patterns during the differentiation of amniotic fluid-derived stem cells into cardiomyocytes, the exact epigenetic mechanisms underlying the cardiomyogenesis of AFSCs and its influencing factors are not still well understood. Therefore, further studies are required to clarify the specific epigenetic mechanisms and the complex molecular networks behind the cardiomyogenesis of AFSCs.

Conclusion

AFSCs are a subpopulation of prenatal stem cells that can easily be isolated from small volumes of AF and stably expanded in long-term cell cultures *in vitro*. These cells are introduced as a new class of highly stable stem cells with intermediate plasticity properties between embryonic and adult stem cell types. Moreover, they are immunocompatible and have anti-inflammatory and differentiation potential into different cell types of all three germ layers. AFSCs exhibited morphologies, phenotypic characteristics, and trilineage mesoderm differentiation potentials similar to MSCs. Moreover, they are highly proliferative cells with a stable karyotype in the long-term cell cultures and with trilineage differentiation potency into cardiomyocytes, endothelial cells, and smooth muscle cells. AFMSCs, as a subpopulation of AFSCs, may be pluripotent stem cells that are less differentiated than BM- and AD-derived MSCs and also their gene expression is almost not influenced by gestational age and culture conditions *in vitro*. Therefore, they can be considered an attractive potent and stable resource for research and therapeutic applications. Nowadays, AFSCs can efficiently differentiate into cardiomyocytes *in vitro* and *in vivo* by different methods and protocols including co-culture approaches, injection or transplantation of the cells into the heart of animals with ischemia/reperfusion injury, treatment with suitable cardiomyogenic induction media and small-molecule chemicals, and the transdifferentiation of AFSCs-derived iPSCs. Therefore, they can be introduced as attractive cells for the treatment of some human cardiac failures, especially neonatal heart disorders.

It has been found that combination treatment with cytokines and GFs, including BMP, IGF, and FGF and the activation of their signaling pathways, is a potentially effective and reliable treatment modality for the differentiation of MSCs into cardiomyocytes. At the molecular level, control of the expression patterns of genes by epi-miRNA mechanisms is critical for the regulation of AFSCs characteristics, especially their differentiation. According to the literature review of the previous studies, we suggest that the epigenetic regulators influencing the global 5'-mC, H3K9me3 and H3K27me3 rates such as DNMT-1/2, HDAC-1/2, EZH2, SUZ12, and BMI1, the stemness markers including OCT4, SOX2, NANOG, KLF4, c-MYC, and REX, miRNAs like let-7a-5p, mir-17, miR-17-3p, mir-21, miR-34c-5p, miR-106b-5p, miR-185-5p, and miR-449a and the cell cycle regulating proteins such as p53, p16, p21, and ATM are the most important factors that affecting the AFSCs characteristics. Furthermore, DNA methylation, the transcription repressive histone marks including H3K9me3 and H3K27me3 on the stemness regulatory genes and also the transcription activation histone marks including H3K4Ac, H3K9Ac, H3K4me3, H4K4Ac, and H4K9Ac on the cardiac-specific genes are critical epigenetic modifications during the differentiation of AFSCs into cardiomyocytes. Therefore, identification and utilizing DNMTs, HMTs, and HATs activators or also the DNMTs and HDACs inhibitors can help to develop more new efficient protocols for the differentiation of AFSCs into cardiomyocytes. Moreover, the most important molecular changes that occur during the differentiation of

AFSCs into cardiomyocytes are including the induction of the ERK signaling pathway, upregulation (GCN5, EZH2, SUZ12, DNMT1, and HP-1α) and downregulation (HDAC-1/2) of the epigenetic modifiers, suppression of HDAC1/2 and stemness markers (OCT4, NANOG, SOX2, and REX1), upregulation of the cell cycle regulators (p53, p21, Rb, and p130), the relative expression of miR-34a and miR-145, and induction of the expression of structural and functional-specific genes of cardiomyocytes (*GATA4, Nkx2.5, cTnT, Nkx2.5, MHC, Myh6, and Tnni3*).

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