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Review Article

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On the Problem of Cardiogenic Differentiation: Extracellular Matrix as an Emerging Clue

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Abstract

It is well known that myocardium fails to regenerate due to the lack of adult cardiomyocyte proliferation. In this regard, a lot of methods for cardiogenic differentiation of different cells are currently developed to provide an alternative source of functionally active cardiomyocytes, including embryonic stem cells, cardiac stem cells, induced pluripotent stem cells and fibroblasts. However, the approaches available show quite a poor result in the efficiency as well as estimation criteria of cardiac differentiation. Many studies are intended to reveal the fundamental mechanisms of cardiogenesis to mimic them *in vitro*, but significant advances in studying of intracellular signaling pathways during heart development are insufficient to produce high efficient methods. Extracellular matrix is another component which is becoming acknowledged as a key player in heart differentiation. However, investigation of its spatiotemporal effects on cardiomyocyte maturation is limited due to the challenges in cell-matrix handling within the heart tissue. We consider that cardiomyocyte primary culture may be a good model for studying the role of extracellular matrix in cardiogenesis, being an easy to manipulate homogenous culture of cardiomyocytes, which synchronically follow rearrangements similar to their embryonic developmental stages. The better understanding of the cell-matrix interactions underlying cardiomyocyte maturation may bring researchers closer to the efficient cardiogenic differentiation.

Keywords

Cardiomyocytes differentiation; Extracellular matrix; Primary culture

Introduction

The myocardium fails to regenerate due to the lack of adult cardiomyocyte (CM) proliferation. In mammals, most of the damages to the heart after myocardial infarction, ischemia, viral infection or other pathological conditions lead to a significant loss of cell mass, which is replaced by scar tissue [1]. In cardiogenesis CMs potently proliferate, but sometime after birth they undergo terminal differentiation accompanied by their permanent cell cycle exit. Postnatal CMs become highly organized postmitotic cells with further growth being associated with hypertrophy [2-6]. Most adult CMs do not divide, but there is still evidence that a small amount of postnatal CMs can enter mitosis. However, cell divisions were shown to occur rarely in the normal adult heart (about 1% per year) [7], which cannot contribute significantly to cardiac regeneration [2,3]. In turn, some researchers suggest self-renewal of the heart with the endogenous precursors of CMs referred to as cardiac stem cells [8]. These cells were identified by the various cell surface markers, such as c-kit and SCA-1 [9]. Cardiac stem cells were described as self-renewing, clonogenic and multipotent cells able to differentiate into all types of cardiac tissue cells and to express antigens and markers of stem cells and endothelial progenitor cells [10]. Some data described isolated cardiac stem cells to differentiate and restore heart function when transplanted into the damage area [11,12]. However, much fewer data can confirm their existence *in vivo*. To date, the functional role of endogenous stem cells in the heart has not been established. The analysis by direct labeling of endogenous c-kit cardiac stem cells *in vivo* showed their negligible percentage in adult myocardium even after injury [13]. Overall, although there are likely to be some cells in the heart tissue that have a limited potential to differentiate into cardiovascular cells when isolated and propagated *ex vivo*, there is no reliable evidence of their role in normal heart homeostasis as well as post-injury regeneration. Similarly, despite the data indicating the presence of mitotically active CMs in the adult mammalian hearts, the percentage of these cells is too small to compensate for the cell death caused by the disease [2,3]. In this regard, a huge amount of research is aimed to differentiate various cells towards CMs to obtain an alternative source of functionally active myocytes.

Methods for Cardiogenic Differentiation of Stem Cells

Different cell types are being used in the *in vitro* CM differentiation methods, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) and endothelial progenitor cells [14-17]. Since adult stem cells show poor potential to produce CMs [18], the main source of differentiated CMs are ESCs and iPSCs. ESCs are derived from the inner blastocyst cell mass at the early stages of embryogenesis. These cells preserve the ability to differentiate in any direction. It has long been known that CMs can be obtained by spontaneous differentiation of mouse ESCs in suspension culture [19]. However, such approach of non-targeted CMs differentiation is little effective and leads to the production of not more than 1% of CMs [20]. Some investigators have modified this method by the centrifugation step in percoll gradient providing up to 70% contracting embryonic bodies [21]. However, they might contain non-beating cells and the percentage of contracting CMs in different culture areas might vary significantly. Another method of ESC coculturing with mouse endoderm-like cells END-2 [22] based on the action of END-2-produced signaling molecules also resulted in a small amount of CMs (typically about 1%) [23].

More effecient methods are based on the targeted differentiation. Such methods typically recapitulate the stages of normal heart embryogenesis and the factors implicated therein. Particularly, in embryogenesis, the successive stages of CMs differentiation depend on the Nodal, bone morphogenetic protein (BMP), Wnt/β-catenin, fibroblast growth factor [24], and retinoic acid signal pathways [25]. Various methods of directed stem cell differentiation towards CMs include manipulating of the Nodal/activin and BMP4 [26] and the Wnt/β-catenin signal pathways [27–29]. Yang and co-authors [28] developed a more efficient protocol for the differentiation of human ESCs, including the sequential stimulation of the Nodal/activin,

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BMP4, and Wnt signaling pathways as the cells undergo certain differentiation stages. This method resulted in about 40% CMs of the total cell population [28]. The use of alternative low molecular compounds to simulate signaling pathways described above was also proposed [30-32]. Despite some advantages in ESC differentiation towards CMs, such methods are still of low efficiency (about 1% to 40% of the total cell population [28,20]) and show different line to line results. Moreover, as for the regenerative medicine the use of ESC-derived CMs is limited by their potential immunogenicity when administered *in vivo*, as well as ethical implications [33].

The development of CM differentiation methods from the induced PSCs (iPSCs) circumvented the above problems. iPSCs are produced by inducing a pluripotent state in mature somatic mammalian cells with a set of transcription factors [34]. iPSCs were first described in 2006, when the Yamanaka group [34] converted adult mouse fibroblasts to iPSCs by retroviral delivery of transcription factors Oct4, Sox2, c-Myc and Klf4 (known as Yamanaka factors). iPSCs were shown to be similar to ESCs in morphology and gene expression [34], including the expression of pluripotent markers and the ability to differentiate into three germ layers [35-38]. Different researchers have shown that iPSC can differentiate towards CMs, with the properties of iPSC-derived cells being comparable with those of the ESC-derived CMs [39]. To improve the efficiency of directed cardiac differentiation of iPSCs, various cytokines, growth factors and low molecular compounds affecting signaling pathways involved in the heart development have been used [40]. Such compounds include ascorbic acid [41-42], retinoic acid [43], pluripotin [44], 5-azacytidine [45], and DMSO [46].

Transdifferentiation Method

Of great importance were the studies showing that mature mammal somatic cells can be converted into functionally active mature cells of another type by transdifferentiation [47]. These data disclosed high plasticity of terminal differentiated cells and offered new approaches in studying the mechanisms underlying cell specialization in development [48]. The method of somatic cells transdifferentiation into CMs includes several approaches, the first being associated with the induction of CMs-specific transcription factors overexpression. In 2010, Ieda and colleagues [49] have shown that the introduction of transcription factors Gata4, Mefc2, and Tbx5 (GMT) into murine cardiac and dermal fibroblasts resulted in the conversion of 20% cells into induced cardiomyocyte-like cells (iCMs) with the expression of cardiospecific proteins after 3 days in culture [49,50]. Similar results were obtained by other researchers using similar sets of reprogramming factors [51-53]. In these cells, activation of cardiac α-myosin heavy chain (αMHC) and cardiac troponin T (cTnT1) genes was observed, as well as increased expression of other cardiospecific proteins such as cardiac α-actin and α-actinin-2. The percentage of cells with the activated αMHC promoter was 20% of the total fibroblast population, whereas the percentage of cells expressing cTnT was only 6% [49]. Most cells with active αMHC showed sarcomeric structures containing α-actinin. However, the authors generally described transdifferentiated CMs as "partially reprogrammed". These cells were similar to neonatal CMs in gene expression profile and electrophysiological indexes. Thus, the differentiation efficiency remained rather low. In addition, these methods were characterized by low reproducibility [54,55]. The method introducing GMT transcriptional factors in stoichiometric amounts appeared to be more efficient [56]. Using a combination of 5 cardiac transcription factors GMT, Hand2, and Nkx2.5 (GMTHN) also resulted in more efficient reprogramming of murine fibroblasts. However, even in this case, most of the *in vitro* differentiated cells were only partially reprogrammed CMs with only few cells developing into contracting CMs [51,57-59]. Other combinations of transcription factors were shown to activate many cardiac genes in fibroblasts, but did not result in contracting CMs [52-54, 60,61].

The second approach relates to the delivery of microRNAs (miRNA), which play an important role in heart development. The miRNA-mediated conversion of mouse fibroblasts into CMs was first described by Jayawardena and colleagues [62] using a combination of 4 miRNAs (miR-1, -133, -208 and -499) known to be expressed in CMs and involved in heart development and function [63-67]. In the reprogrammed cells, CM markers and sarcomere organization of the contractile apparatus were detected along with mechanical contractions of the cells. An additional step of culture treatment with Janus kinase (JAK) inhibitor increased the efficiency of mouse fibroblasts transformation into CM-like cells *in vitro* [62]. However, although the cells differentiated with such a method were stained for CMs markers, including MHC, cardiac troponin I (TNNI3), and α-actinin, spontaneous contractions were observed in only 1 to 2% of the cells [62].

Other approach called the "epigenetic instability" combines several methods described previously. It is based on the induction of the intermediate pluripotency state in fibroblasts followed by the directed differentiation towards CMs [68]. This method involves overexpression of transcription factors used for iPSC production (Oct4, Sox2 and Klf4) in embryonic fibroblasts followed by their treatment with the low molecular JAK inhibitors and subsequent cultivation in cardiogenic medium with BMP4 [68,69]. Differentiated cells were stained for CMs markers, such as cTnT (approximately 40% of cells), myosin heavy chain and α-actinin. Interestingly, in these cells only the atrial isotype of the myosin light chain (MLC-2α) was detected, which indicates subtype specialization of CMs obtained. In some colonies, contractions were observed [68,69].

Limitations of the Cardiogenic Differentiation Methods

In spite of the advances achieved in cardiogenic differentiation, the current methods have a number of limitations associated with the immature phenotype of the resulting cells and their heterogeneity.

To date, all available protocols for cardiogenic differentiation of stem cells result in a mixture of different CM subtypes, i.e. ventricular, atrial and conducting CMs (see, for example, [70-73]). Although the percentage of ventricular CMs increases over time [74,75], general heterogeneity of the population makes it difficult to use these cells for *in vitro* studies, and leads to a pro-arrhythmogenic effect when administered *in vivo* [76,77]. To address this challenge, various growth factors have been used, regulating specialization of CM subtypes. Such factors include, for example, neuregulin stimulating maturation of ventricular CMs [72].

For iPSCs or iCMs, the population heterogeneity is complicated by epigenetic modifications that can limit the potential of these cells to differentiate towards CMs. For example, mouse iPSCs at the early passages retain some epigenetic modifications of the cells from which they were derived. Such iPSCs differ in their ability to differentiate [78] resulting in a culture containing both CMs and undifferentiated cells, which limits its *in vitro* application and can lead to a tumorigenity when administered *in vivo*. Thus, a homogeneous culture of differentiated CMs or at least a well characterized combined culture of different cardiac cells is still elusive.

Another significant limitation is that most CMs derived from stem cells, as well as produced by transdifferentiation, have a so-called "immature phenotype", i.e. differ from CMs of adult myocardium. At the early stages, stem cell-derived CMs are much smaller in size and exhibit characteristics of embryonic CMs, such as amorphous form and short, poorly organized sarcomeres [79-82]. In addition, the pattern of gene expression in these cells corresponds to the stages of the embryonic development of the heart [20,83]. Although differentiated CMs were shown to increase in size and acquire a more typical morphology [80,82,84] and a more mature contractile apparatus with time [74-75,80], even at the late stages of cultivation (about 180 days in culture) these cells do not correspond to adult CMs in electrophysiology and gene expression [85]. Some data indicate a decreased mass of mitochondria in stem cell-derived CMs and the absence of T-tubules [86-88]. Similarly, the cells produced by transdifferentiation do not correspond to adult CMs in morphology, gene expression profile, and electrophysiological indices, and are generally referred to as "CM-like cells" [54,89].

Another challenge is the ambiguous interpretation of CMs differentiation results. Despite the "mature" and "immature" CMs phenotype referred to in many studies, conventional markers for identifying differentiated CMs, as well as the status of their maturation, are absent [49,90-92]. Although a large number of cardiospecific proteins with characteristic expression patterns in cardiogenesis are widely used by researchers to identify differentiated CMs, many of them tend to alter in stress or pathological conditions [93]. For example, the α-myosin heavy chain gene is highly specific for CMs, however, in heart disease, myosin isoform pattern changes [94]. Heart failure activates genes specific for embryonic development, such as atrial natriuretic factor, βMHC and skeletal actin genes [93,95]. In this regard, the results obtained with such unstable markers should be interpreted with caution [96]. Another widely used marker for cardiomyocyte differentiation is cardiac troponin T (cTnT) [97]. However, the expression of cTnT has also been shown in other cells, such as smooth muscle cells [98], which limits its use as a CMs marker.

Bedada et al. offered the ratio of cardiac troponin I isoforms as a valuable marker of CM maturation status [93]. In the mammalian heart, there are two troponin I (TnI) isoforms, encoded by two different genes and sequentially expressed in the development [94,99]. The slow skeletal troponin I (ssTnI) TNNI1 gene is expressed in sarcomeres of embryonic CMs. At the late stages of embryonic development and/or at the early stages of postnatal development, its expression is suppressed, and the TNNI3 gene of adult cardiac troponin I (cTnI) is up-regulated. Thus, in the adult myocardium, only the cTnI protein is detected with the absence of ssTnI [94,99]. Importantly, stress and pathological conditions do not influence the dynamics of ssTnI and cTnI isoforms expression [94,100,101], in contrast to reversible markers of cardiomyocyte differentiation, commonly used by the researchers [95]. The analysis of the ssTnI and cTnI isoforms ratio showed that the expression of troponin I isoforms in murine ESC-derived CMs was similar to the pattern of their expression *in vivo*. However, in the case of human iPSC-derived cells, CM maturation was significantly delayed compared to normal cardiac development [83], with the cTnI being only 2% of total troponin I even by 9.5 months in culture [83].

Thus, although many authors refer to the high yield of CMs obtained with differentiation approaches, the methods for evaluating of CM differentiation status are generally ambiguous, and the actual percentage of the functionally active CMs is much lower. For example, the authors describing the cultures containing 82% to 95% CMs identified differentiated cells by the expression of cardiospecific transcription factors and cardiac troponin T staining. However, study results indicate that contractions were observed only in some areas of the culture [31]. In another study, despite the various cardiospecific markers detected, the contracting CMs were completely absent from the culture [89]. One more research described spontaneous contractions only in 1% to 2% of the cell population, whereas such markers as MHC, cardiac troponin I (TNNI3) and α-actinin were detected in a significantly larger cell percentage [62].

Extracellular Matrix in CM Differentiation

Despite a lot of efforts targeting cardiogenic conversion of different cells, the use of differentiated CMs is still limited due to the lack of a standard method providing for a homogeneous culture of mature, functionally active cardiac cells. Therefore many studies are intended to reveal the key mechanisms of the *in vivo* CM differentiation to mimic them *in vitro*. Despite significant advances in studying of intracellular signaling pathways during heart development, much less is known about the role of extracellular matrix (ECM) in cardiogenesis. This gap in our knowledge is likely to be accounted for the difficulties associated with the *in vivo* investigation of cell-matrix interactions in heart tissue. At the same time, heart ECM is emerging as a powerful regulator of migration, proliferation and differentiation of cardiac cells [102]. It should be noted that some methods include ECM as a scaffold to enhance differentiation of different cells towards CMs. For example, commercial matrixes Matrigel and Geltrex have been demonstrated to support the early cardiac program in direct cardiac reprogramming methods [68]. Recent study has shown that biological cardiac matrix facilitated sarcomere alignment in human iPSC-CMs [83]. Moreover, a fibroblast-derived ECM called cardiogel was shown to improve maturation of mouse ESC-derived cardiomyocytes [103]. Native ECM, obtained by heart decellularization, was also described to maintain the differentiated state of iPSC-CMs and their capability of forming tissue segments [104]. Besides its supplementary role some studies indicate the direct matrix influence on cardiac differentiation in the absence of other stimulating factors. Bone-marrow-derived stem cells from adult mice grown on cardiogel showed increased cardiomyogenic differentiation without specific chemical induction [105]. Interestingly, some authors credit several effects of chemical cardiogenic factors not with their direct action, but with their stimulation of matrix components production. For example, several groups have suggested that ascorbic acid enhances differentiation of ESCs and iPSCs into cardiomyocytes through induction of collagen synthesis [106,107]. Some data indicate ECM identity to be important for CMs differentiation. The expression of cardiac myosin heavy chain (cMHC) and cardiac troponin I (cTnI) in murine ESC-derived CMs was high in cells cultured on the heart ECM compared to those cultured on the liver ECM [108]. MSC-derived cardiomyocyte-like cells showed enhanced expression of cardiospecific markers GATA4 and Nkx2.5 when cultured on a collagen V as compared to a collagen I matrix [109]. According to another study fibrin gels supported cardiac differentiation in cardiac reprogramming method, whereas Matrigel and collagen I gels were poorly efficient [107]. Besides the ECM identity, its mechanical properties have been implicated in cell fate directing [110,111]. ECM density and/or stiffness was shown to regulate embryonic and neonatal cardiomyocyte structure and function [112,113] as well as early embryonic or embryonic stem-cell derived cardiomyocyte differentiation [114-117].

In spite of the fact that current approaches using ECM show better results, they still fail to provide an appropriate culture of functioning CMs. Matrix involvement in CMs differentiation appears to be much more complicated in embryonic heart tissue than is achieved by the crude differentiation methods using different ECM scaffolds. The data from the last decade have shown that myocardium ECM is finely regulated dynamic system and is capable of fast reacting to the alterations in heart load. Some authors have shown that ECM composition and distribution alters during cardiogenesis and these alterations may be crucial for cell differentiation process [118-120]. Moreover, the matrix stiffness was also shown to alter in cardiogenesis with the cardiac tissue becoming less compliant during development [117].

Interactions between cells and ECM are provided by integrin receptors, which are transmembrane heterodimers connected with the ECM components by their extracellular domain and with cell contractile structures via intracellular domain. Therefore integrins are able to transmit mechanical signals from ECM to the anchored cells and transfer them into the intracellular stimuli [121,122]. This process is known as mechanotransduction, which differs from those involved in adhesion [123]. Some studies imply that neonatal and adult myocytes have different integrin receptors suggesting that integrin expression is developmentally regulated [112]. Therefore complex interactions between the ECM molecular composition and the integrins expression patterns are likely to coregulate contractile apparatus remodeling during cardiac development with spatiotemporal control [112].

To improve differentiation of different cells towards CMs the ECM dynamics involved in cardiogenesis are to be extensively studied and strongly considered in method developing. Due to the challenges with the *in vivo* estimation of cell-matrix interactions, to discover the chronological relationship between the matrix changes and contractile apparatus maturation during heart development a good *in vitro* model is needed.

CMC in Primary Culture

It is well known that neonatal as well as adult CMs are readily transferred to the culture system resulting in a homogenous culture of functionally active CMs. However, CMs in long-term culture were shown to undergo reversible rearrangements of their contractile apparatus with conversion of typical myofibrils into the structures of non-muscle type and the loss of contractility [124-127]. These rearrangements are accompanied by the transient expression of α-smooth muscle actin in CMs, which is normally restricted to vascular smooth muscle cells and myofibroblasts. Interestingly, the following recovery of CM myofibrillar apparatus and contractile activity occurs with new myofibrils formation and re-expression of cardiac actin [128-130]. Similar actin isoform switch is well described in early cardiogenesis, as well as during stem cell differentiation towards cardiomyocytes [131,132], where α-smooth muscle actin is transiently expressed and replaced by sarcomeric actin isoforms as development proceeds [125,133-136].

These data allow us to consider the rearrangements observed in CM primary culture as dedifferentiation process followed by the maturation from embryonic to adult CM phenotype.

Previously we have shown that dynamics of CMs contractile apparatus in primary culture are strongly dependent on the ECM [127]. Our recent data indicate that rearrangements of contractile apparatus are accompanied by ECM synthesis by CMs themselves [127,130]. Significantly, the ECM accumulation goes along with α-smooth muscle actin downregulation and precedes the upregulation of α-cardiac actin expression and myofibrillar apparatus assembly, suggesting the feedback loop between the ECM dynamics and contractile apparatus maturation in the cultivated CMs [130].

In the light of these data, we suggest that CM primary culture can be a good model for studying the ECM involvement in cardiogenesis, being a homogeneous culture of CMs, which synchronically undergo rearrangements corresponding to the stages of embryonic development.

In conclusion, the differentiation of different cells towards CMs is significant and promising trend. However, current methods are still of low efficiency. Meanwhile, the important role of ECM in the heart development is becoming apparent through the last decade offering a new approach to optimize cardiac differentiation methods via matrix cues. Better understanding of the cell-matrix interactions underlying CM maturation may allow for the management of this process and bring researchers closer to the efficient CM differentiation.

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