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# In vitro differentiation of mouse embryonic stem cells into cardiac cells

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## Abstract

*In its evolution, medicine has imposed a batch of experimental systems that have tried to explain the miracle through which a fertilized cell transformed in blastocyst lies at the basis of all the specialized tissues that form an organism. It seems that the answers derived from these studies intensified the number of medical questions, engendering a new research theme: the embryonic stem cells.*

*Embryonic stem cells are derived from preimplantation blastocyst, they are pluripotent cells and they are able to differentiate to different cell lines. This review aims to structure different differentiation protocols most commonly used by different specialists in the field, specifying the role of inducer substances and of the specific methods and substrates used.*

**Keywords**: mouse, embryonic stem cells, pluripotent, cardiac differentiation

## 1.Introduction

Embryonic stem cells (ESCs) are undifferentiated pluripotent cells (derived from the inner cell mass of blastocyst stage embryos and can be propagated *in vitro* indefinitely [1,2,3], maintaining long-term self renewal and the capacity to give rise to all cell types in the adult body when subjected to the appropriate conditions [4].

ESCs can be maintained in the presence of leukemia inhibitory factor (LIF) or in co culture with mouse embryonic fibroblasts (MEFs), [5] without losing their pluripotency and their stable karyotype [6]. In the absence of LIF or MEF feeder layers or cultivated on a non-adhesive substrate, ESCs differentiate spontaneously and can generate multicellular/three-dimensional (3D) aggregates called embryoid bodies (EBs) [6,7]. The formation of embryoid bodies (EBs) is the principal and the first step in the differentiation of embryonic stem (ES) cells [8]. This structure facilitates multicellular interactions, in which cell-cell contact exists and gap junctions may be established [9,10]. Because of the important role the EBs played in the *in vitro* differentiation system of ES cells, the quality of EBs formed from ES cells affects the induction efficiency of derivatives from the EBs in a subsequent differentiation culture [11,12]. Generally, the formation and differentiation of embryoid bodies includes the following two main categories. One is scaffold-free three-dimensional culture system, including bioreactors stirred-suspension culture, round-bottomed conical tube, and so on. Although the scaffold-free systems are simple and produce large numbers of EBs, it is difficult to carry out ESC three-dimensional differentiation and tissue formation research without adding three-dimensional materials. The other category is three-dimensional scaffolds-culture system, including microcapsules, PLGA [12].

## 2. Preliminary steps for differentiation of murine embryonic stem cell: Embryoid bodies formation

ESCs cells are viewed as a promising cell source for cell transplantation because of their unique ability to give rise to all somatic cell lineages [2,13,14]. In culture condition, when factors that maintain the pluripotency of ES cells are removed, ES cells spontaneously differentiate into derivatives of the three embryonic germ layers: the mesoderm, the endoderm, and the ectoderm [15]. The formation and early differentiation of embryoid bodies (EBs) is a principal step in the differentiation of ESCs *in vitro*. An EB can consist of ectodermal, mesodermal, and endodermal tissues, which resumes many aspects of cell differentiation during early mammalian embryogenesis and differentiate into derivatives of all the three germ layers [9,10,16]. EBs play an important role in the differentiation of ES cells into different kinds of cells in vitro and proved valuable for genetic studies of tissue differentiation [17]. Generally, there are three methods to induce EB formation [18]: in hanging drops [8,17,19,20] in liquid suspension culture in bacterial-grade dishes [8,19,21,22] and in methylcellulose semisolid media [23,24]. Each of the methods has own peculiarity, in which ES cells are cultured under various conditions [8]. The quality of formed EBs affects further differentiation occurring in EBs afterwards; this feature differs between EBs depending on the methods, because culture conditions such as cell density, culture period, and culture vessel are not the same. Heterogeneity in the quality of EBs may have detrimental effects on the synchronism of differentiation. To guarantee the homogeneity of EBs a reliable method is required for EB formation with reproducibility [8].

Homogeneous EBs are reproducibly formed from a predetermined number of ES cells. Hanging drops culture is the most frequently used in mouse ES cell differentiation, but not so much in human ES cell differentiation [25,26,27]. Spinner flasks [8,28-33] or bioreactors [8,31,34,35] are amenable to process control strategies for a large-scale production of EBs. Generally, hanging drop culture is followed by suspension culture in bacterial dishes [8,36-41]. Suspension culture in bacterial dishes is the most basic method that is also applicable to EB formation induction from small clumps of human ES cells [10,44-47] not only to that from single mouse ES cells. Methylcellulose culture is preferable to hematopoietic differentiation [8,23,48,49]. The common disadvantage of these methods is the lack of support from extra-cellular matrix, which plays an important role in cell growth and development [12]. Embryoid bodies can be classified as simple or cystic according to their stage of differentiation [23,50-52]. However, the complex interactions that control the transition of ectoderm to visceral and parietal endoderm in the post implantation embryo followed by the formation of mesoderm at the gastrulation stage (days 3-7 post coitum) are only beginning to be defined [52].

These aggregates play a key role as an *in vitro* differentiation of ES cells, and might help elucidate the processes taking place at the beginning of embryonic development, such as lineage determination and differentiation [53].

## 3. Directed differentiation of mouse embryonic stem cells – cardiac differentiation

Stem cells have the remarkable potential to develop into many different cell types. When a stem cell divides, each new cell has the potential to either remain a stem cell or become another type of cell with a more specialized function [54].

The enhancement of differentiation towards a specific lineage [55-57] can be achieved by the followings: activating endogenous transcription factors; transfection of ESCs with ubiquitously expressing transcription factors; exposure of ESCs to selected growth factors; or co culture of ESCs with cell types capable of lineage induction. ESCs may be induced to form the lineage of interest by a combination of growth factors and/or their antagonists, [57,58],

signaling molecules, and extracellular matrix (ECM) proteins constituting the developmental 'niche' in which the cells exist [59].

*In vitro* differentiation is commonly induced by withdrawing leukemia inhibitory factor (LIF), through formation of aggregates known as embryoid bodies (EBs). EBs essentially contains a broad spectrum of cell types representing derivatives of the primary germ layers and morphologically resembles the extra embryonic yolk sac.

Mesoderm-derived lineages, including the hematopoietic, vascular, and cardiac, are among the easiest to generate from ES cells and have been studied in considerable detail [57].

To induce cardiac differentiation different factors can be added into medium such as retinoic acid [69], dimethylsulfoxide, co-culture with a mouse visceral endoderm-like cell line (END-2) [60], with [61] or without serum, 5-azacytidine, and down regulation of Notch1 signaling [62]. Multiple studies describe the effect of different growth and differentiation factors that have the ability to induce cardiomyocyte differentiation from stem cells. Among growth factors transforming growth factor-β (TGF-β) [63], insulin-like growth factor (IGF)[64,65], fibroblast growth factor (FGF) [66,67], vascular endothelial growth factor [68], are often used, but also erythropoietin [69], oxytocin [70], retinoic acid [70,71], dimethylsulfoxide (DMSO) [72] and ascorbic acid [73] are frequently included into differentiation media [74] (Table 1).

**Bone morphogenic proteins**

Bone morphogenic proteins (BMPs), members of TGF-β super family [75], are dimeric mature proteins with notable value in cardiac induction [76] and play an important dimeric mature proteins with notable value in cardiac induction [76] and play an important role in early embryogenesis as well as in the inhibition and induction of cell di□erentiation, including cardiac myocytes [77,80]. Both BMP2 and BMP4 added to media of explants cultures induce cardiac diferentiation in stages 5–7, anterior medial mesoderm, a tissue that ordinarily does not manifest cardiogenic potential [81]. BMP2 exposed to ES–EBs cell systems enhances the diferentiation to beating cardiac myocytes, a change inhibited by noggin [79, 82]. Noggin, BMP antagonist protein inhibits precardiac mesoderm diferentiation, suggesting that BMP activity is required for cardiac diferentiation [81].

BMP-4 treatment in suspension period of EB culture system had an inhibitory effect on cardiomyocyte differentiation from ESCs, decreased the total percent of contracting EBs and reduced the percent of cardiomyocytes per EBs [83].

**Transforming growth factor-β**

The transforming growth factor-β (TGF-beta) super family comprises nearly 30 growth and differentiation factors that include TGF-betas, activin, inhibin, and bone morphogenetic proteins (BMPs). Multiple members of the TGF-beta super family serve key roles in stem cell fate commitment [84]. TGF-β has been shown to induce cardiac di□erentiation from ES cells as well as regulate cell growth, diferentiation, and migration during embryonic development [85,86,88]. Three di□erent isoforms of TGF-β; β-1, β-2, and β-3, have been identified in mammals [88]. The TGF- β isoforms of knockout mice have been shown to be phenotypically and functionally distinct for heart development.

Dinender K. et al. research shows that TGF-β or –β3 treatment of mouse ES cells with TGF- β 2 isoform significantly increased embryoid body (EB) proliferation as well as the extent of the EB outgrowth that beat rhythmically. TGF-β isoforms have also been shown to generate distinguishing e□ects in vitro as well as ex vivo. For example: TGF-β 1, and not TGF-β-2, inhibits proliferation of hematopoietic progenitor cells [89]; bovine aorta endothelial cells (BAECs) [90]; TGF- β-1, and not TGF- β-2 also significantly inhibits cell migration of BAECs [90]; TGF- β-2 and not TGF- β-1, plays a role in mesoderm induction studied in amphibian explants [89]; TGF-β-2 and not TGF- β-3 has been shown to induce mouse epithelial–mesenchymal transformations in collagen gel explant cultures [91].

**Fibroblast growth factor**

It is a member of the family of heparin-binding growth factors that bind tyrosine kinase receptors. Fibroblast growth factors (FGFs) are polypeptides comprising a family of 22 members [92]. In the heart, FGF-2 expression was shown to be up regulated after cardiac injury, such as ischemia/reperfusion, or in the process of cardiac remodeling [93] FGF-2 has been implicated in cell proliferation, survival, and differentiation [94] and plays a role in driving mesodermal cells to the cardiogenic lineage during embryogenesis [95] FGF2 exposed ES–EB cell system enhanced cardiomyogenic di erentiation [96].

**Hepatocyte growth factor**

Hepatocyte growth factor (HGF) is a pleiotropic cytokine promoting proliferation, migration and survival in several cell types. It is a potent mesodermal-derived mitogen involved in differentiation, proliferation, migration, and survival of different cell types. HGF and its receptor, the proto-oncogene c-met, are expressed not only in full differentiated cardiac cells but also in myocytes during early cardiogenesis and it has been speculated that HGF might be involved in cardiac development [97, 98]. HGF plays a pivotal role during embryogenesis being involved in several steps of organogenesis. HGF is highly transcribed and specifically co-expressed with its receptor in the restricted period of time that will give origin to cardiac myocytes and formation of the functioning heart [98], indicating a probable role in coordinating the complex program of cardiac cell differentiation. These findings have raised the idea of using HGF in combination with other factors or cytokines to manipulate stem cells differentiation towards a cardiac lineage [97].

**Vascular endothelial growth factor**

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen with potent permeability properties [99]. This growth factor exists in several isoforms; the most abundant form present in most tissues is VEGF165. The different isoforms exhibit differences in biologic function. During development, VEGF is expressed in multiple embryonic and fetal tissues, with the highest levels found in the lung, kidney, and heart [99]. VEGF binds to its tyrosine kinase receptors, FMS-like tyrosine kinase-1 (Flt-1) and fetal liver kinase-1 (Flk-1). Inactivation of VEGF during early embryonic development results in myocardial defects [100].

**Insulin and insulin-like growth factors**

Insulin/insulin-like growth factors and their intracellular downstream target protein kinase Akt are known to protect many cell types from apoptosis and to promote proliferation, including hESC-derived cardiomyocytes [101]. Insulin-like growth factor I (IGF I) has been shown to be essential for normal embryonic growth in mice [102] and for the formation of a functional heart [103]. Gene expression for insulin receptors and insulin-like growth factors (IGFs) with their cognate receptors has been documented from the early phases of murine embryonic life [104].

Not only is IGF1 essential for normal embryonic growth and development in mice [102], but severe deficiency may affect the functional maturation of the cardiovascular system [105]. Antin et al. have suggested that insulin and IGFs may promote avian cardiac development *in vivo* by both autocrine and paracrine mechanisms. The addition of recombinant IGF1 to a pre transplantation murine embryonic stem cell suspension was associated with increased *in vivo* expression of a cardiomyocyte phenotype and functional improvement [106]. Thus, besides their well described role involving linear growth, glucose metabolism and organ homeostasis, these pleiotropic hormones appear to be involved in early phases of cardiogenesis.

**The extracellular matrix**

The extracellular matrix (ECM) was found to be a helpful means to provide some crucial physical signals to influence major intracellular pathways and thereby directing

proliferation, differentiation, cell metabolism and plays a significant role in controlling cellular behavior [107-109].

Matrix composition has an important role in ES differentiation and influences their behavior towards preferred lineages. A number of natural materials have been used to support the differentiation for hESCs that include agarose, alginate, hyaluronic acid, gelatin, fibrin glue, collagen derivatives, and cellular tissue matrices [74]. Collagen is the main component of native ECM and cells interact with collagen through integrin binding-mediated interactions. The scaffolds based on the collagen could provide physical supports; furthermore, its favorable flowability benefits the cell migration, aggregation and the formation of three dimensional structures and has low immunogenicity.

The addition of fibronectin to the collagen gel preferentially stimulated ES cell differentiation into endothelial cells, leading to vascularization, while the addition of laminin favored ES cell differentiation into beating cardiomyocytes [109].

Gelatin is a porous denatured collagen scaffold, and it has been used for tissue engineering applications due to its biocompatibility [112].

**Table 1.** Growth factors for embryonic stem cells differentiation

| GROWTH FACTOR | CELL TYPE | CELL TYPE DEVELOPED | DIFFERENTIATION CONDITION | REFERENCE |
|---|---|---|---|---|
| Bone morphogenic proteins | ESCs | Osteoblast | BMP-2 | R.F. Pereira et al., 1995, J. Kramer et al., 2000, A.C. Perkins et al., 1998, P. Bosch et al., 2000, G. Winnier et al. 1995,T.M. Schultheiss et al. 1997, F.M. Masoumeh et al. 2007 |
| | | Chondrocyte (cartilage-forming cell) | BMP-4 | |
| | | HSC and erythroid | BMP-2, culture on collagen substrate Interleukin-6, absence of LIF | |
| | | Osteoclast, osteocyte, osteoprogenitor | BMP-4, Dexamethasone, Vitamin $D_3$ | |
| | | Ventral mesoderm formation | BMP-2 and BMP-4 | |
| Transforming growth factor-β | ESCs | Skeletal muscle | TGFβ , retinoic acid, β-mercaptoethanol, ES co-culture with stromal cells | H.G. Slager et al., 1993, J. Rohwedel et al., 1994, K. Kitisin et al. 2007, R.W. Pelton et al. 1991, R.J. Akhurst et al. 1990, A.S. Boyer et al. 1999, D.K. Singla et al. 2005, M. Ohta et al. 1987, R. Merwin, et al 1991, T.D. Camenisch et al. 2002 |
| | | Cardiomyocyte | TGFβ, bFGF, BMP-4 | |
| Fibroblast growth factor | ESCs | Embryoid bodies with three germ layers: endoderm, mesoderm, ectoderm | Leukemia inhibitory factor, Basic fibroblast growth factor | M.J. Shamblott et al., 1998, J.N. Reynolds et al., 1996, F. Doetsch et al., 1999, B.M. Johansson et al., 1999, O. Brustle et al., 1999, N. Lumelsky et al., 2001, K.A. Detillieux et al. 2003, R.V. Nathalie et al. 2005 |
| | | Astrocyte, neuron, oligodendrocyte | Basic fibroblast growth factor Epidermal growth factor | |
| | | Pancreatic islet-like | Serum-free media , absence of feeder-cell layer,bFGF, nicotinamide | |
| Hepatocyte growth factor | ESCs | Cardiac myocytes | HGF | R. Cristiana et al. 2007, D.A. Rappolee et al. 1996, P. Yogesh et al. 2000 |
| | | Placental development | HGF, FGF-4, IGF | |
| Vascular endothelial growth factor | ESCs | Endothelial, smooth muscle, vascular, progenitor | Collagen-IV matrix, -LIF, VEGF | J. Yamashita et al., 2000, M. Drab et al., 1997 |
| | | Smooth muscle | Retinoic acid and db-cAMP, culture over collagen IV matrix, VEGF | |
| Insulin-like growth factors | ESCs | Cardiomyocytes | IGF, FGF, VEGF | C. Freund et al. 2008, B.L. Powel et al. 1993 D. Ioannis et al. 2006, H. W. Ping 2001 |

Matrigel, which is extracted from the basement membrane of the Engelbreth- Holm-Swarm tumor, contains laminin, type IV collagen, heparan sulfate proteoglycan, entactin, nidogen and so on. All of these components are biologically active and stimulated the growth and differentiation of certain cells [109]. The combination of the collagen and Matrigel could provide not only physical supports for ES cells development and differentiation, but also the necessary components of extra-cellular matrix.

**Small molecules**

In addition to growth factors and cell-secreted morphogenetic factors, the fate of stem cells can be regulated by small cell permeable molecules such as dexamethasone, vitamin C [111,112], sodium pyruvate, thyroid hormones, prostaglandin E2, dibutryl cAMP, concavalin A, vanadate, retinoic acids [71] dimetylsulfoxid oxytocin. Recently, new biomolecules, in the form of small molecules, have been investigated as a repertoire of differentiation-inducing factors to alter stem cell fates. Ding et al. screened a variety of small molecules for their ability to modulate differentiation of ES cells into various tissue- specific cells [74]. Such small molecules play important roles during embryogenesis and may be used to direct or control the differentiation process of ES cells. As the identification of these molecules and their roles in stem cell biology becomes well understood, they can be incorporated into tissue engineering scaffold design so as to harness their beneficial effects for lineage-specific differentiation and tissue development [74].

**Coculture method**

Coculture methodologies have also been used to produce differentiated cardiomyocytes from hESCs. Mummery and colleagues [60,61] showed that 15–20% of cultures of hESCs grown with the mouse visceral endoderm cell type (END-2) will form beating heart muscle colonies, and this has been substantially increased in more recent experiments. Beating heart muscle cells derived from hESCs express cardiomyocyte markers including α-myosin heavy chain, cardiac troponins, and atrial natriuretic factor as well as transcription factors typical of cardiomyocytes, *e.g*., Nkx2.5, GATA4, and MEF3 [44,57,61]. These cells respond to pharmacological drugs, and the action potentials of cardiomyocytes produced in this system most commonly resemble that for human fetal left ventricular cardiomyocytes but are distinctly different from those of mouse cardiomyocytes [61,113]. The hESC-derived cardiomyocytes are capable of integrating apparently normally when transplanted into rodent and porcine heart muscle, forming gap junction connections between hESC myocytes and the recipient mouse adult cardiomyocytes [113].

## 4. Conclusions

The development of cellular and tissular therapy from the last few years is realized on the basis of some studies focused on the stem cells biology. These therapies require a high number of stem cells, uninvasive purification and isolation methods and also an increased capacity of expansion (proliferation) and the control of the differentiation.

The stem cells therapy requires reproduction conditions in order to obtain stem cells so that the cells constantly present the same characteristics for a successful differentiation, transplantation and engraftment.

Embryonic stem cells (ESCs) can differentiate into all somatic cell types, thereby providing a robust cell source for regenerative medicine therapies. Cardiomyocyte differentiation in mEBs resumes the sequential expression of cardiac genes observed in the mouse embryo *in vivo*. Proper assessment and identification of substances inducing the growth factor or factors with maximum effect is of major importance for inducing differentiation of mouse embryonic stem cells. Multiple studies describe a multitude of substances that can be used to induce cardiac differentiation. But recent studies indicate a

major effect of mixtures of substances or growth factors for obtaining a high percentage of differentiated cells.

The international studies made so far in the domain of embryonic stem cells attested the characteristics of these cells that nowadays became a very important instrument of the regenerative medicine. In Romania there are only a few studies that follow this research direction and they are prevalently pointed to the elucidation of adult stem cells particularities. Therefore the studies in the field of embryonic stem cells have a major importance.

The importance of this theme is immensurable, offering new life hopes, both for the researchers and for the patients, given the possibility of using these cells in aesthetic and regenerative medicine.

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