Effect of bone morphogenetic protein-4 (BMP-4) on cardiomyocyte differentiation from mouse embryonic stem cell

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Abstract

The present study was designed to evaluate the effect of BMP-4 on mouse embryonic stem cells (ESCs)-derived cardiomyocyte. Cardiac differentiation of the mouse ESCs was initiated by embryoid bodies (EBs) formation in hanging drops, transfer of EBs to the suspension culture and then plating onto gelatin-coated tissue culture plates. BMP-4 was added to culture medium throughout the suspension period. Cultures were observed daily with an inverted microscope for the appearance of contracting clusters. At the early, intermediate and terminal stages of differentiation, the chronotropic responses of cardiomyocytes to cardioactive drugs were assessed, and the cardiomyocytes immunostained for cardiac troponin I, desmin, α-actinin and nebulin. The contracting clusters were isolated for ultrastructural evaluation, at day 14 after plating. Moreover, total RNA extracted from contracting EBs of early and terminal stages of differentiation were examined for oct-4, α- and β-myosin heavy chain, myosin light chain-2V and atrial natriuretic factor expression.

The BMP-4 treatment resulted in a decrease in the percent of beating EBs and the percent of developing cardiomyocytes per EBs. As a whole, the chronotropic responses of beating cardiac clusters to cardioactive drugs in control group were better than BMP-4 treated group. The cardiomyocytes of both groups were positive immunostained for applied antibodies except for nebulin. Moreover, in the BMP-4 treated group, the ultrastructural characteristics and cardiac-specific genes expression were all retarded in the terminal stage of cardiomyocytes development.

In conclusion, BMP-4 had an inhibitory effect on cardiomyocyte differentiation from the mouse ESCs in terms of ultrastructural characteristics, genes expression and functional properties.

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1. Introduction

Developmental fate of differentiating stem cells depends on the complex ‘cocktail’ of growth factors, signaling molecules, and extracellular matrix (ECM) proteins constituting the developmental ‘niche’ in which the cells exist [1]. Understanding these factors is an initial step for directing stem cell differentiation toward a desired phenotype. In recent years, some growth and differentiation factors have been shown to induce cardiomyocyte differentiation from stem cells, including transforming growth factor-β (TGF-β5) [2], insulin-like growth factor (IGF) [3,4], fibroblast growth factor (FGF) [5,6], erythropoietin [7], oxytocin [8], retinoic acid [9,10], dimethyl sulfoxide (DMSO) [11] and ascorbic acid [12]. Nevertheless, effect of some growth factors on cardiac differentiation of ESCs remained to be clarified. Moreover, the challenge about some growth factors such as bone morphogenetic proteins (BMPs) is continuing and the exact role of them in cardiogenesis is under more evaluation.

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As known from in vivo studies, BMPs, members of TGF-β superfamily have a notable value in cardiac induction [13].
BMP-2 and BMP-4 induce ventral mesoderm formation during embryogenesis [14]. As reviewed by Zaffran and Frasch [15], in chick and *Xenopus*, solid evidence for a direct role of BMPs in cardiac induction has been obtained. In the chick, the expression of *Bmp2*, 4, and 7, includes the anteroateral region of the embryo which overlaps with the precardiac region that expresses *Nkx2-5* and *Gata4* [16,17]. Ectopic application of BMP-2 or BMP-4-releasing implants in vivo causes ectopic induction of *Nkx2-5* and *Gata4*, although not of terminal differentiation markers, and the exposure of tissue explants to soluble BMP-2 or-4 induces both the early cardiac regulators and also terminal differentiation of cardiac tissue [16–18]. In vertebrates, BMP signals are likely to provide direct inputs in controlling several different cardiac regulatory genes [17,19–22]. Moreover, the heart-inducing activity of BMP signaling has been further confirmed by studies using inhibitors of BMP signaling [17,20,23,24].

In spite of previous reports, few studies have been carried out to determine the effect of BMP signals on the cardiomyocyte differentiation from the ESCs. Recently, Schuldiner et al. [9] showed that some growth factors such as BMP-4 allow or induce differentiation of ESCs into ectodermal and mesodermal cells. Also, Behfar et al. [2] reported that stimulation of ESC-derived EBs with BMP-2 and TGF-β1 resulted in increased cardiac differentiation with a significant increase in cardiac areas and enhanced myofibrillogenesis. Also, when stem cells were grafted onto postmitotic isolated ventricular, cardiomyocytes had the ability to differentiate into ventricular myocytes and beat in synchrony with host cells. This process was significantly enhanced by stem cell priming with TGF-β1 or BMP-2 in vitro, and disruption of the TGF-β1/BMP by Noggin prevented differentiation of ESCs. More recently, Yuasa et al. [25] showed that the BMP antagonist Noggin transiently but strongly expresses in heart forming area and functions in cardiomyocyte differentiation. Moreover, they reported that inhibition of BMP signaling in the undifferentiated or immediate phase of ESC differentiation is crucial for cardiomyocyte differentiation. In the present study, we evaluated the effect of BMP-4 on cardiomyocyte differentiation from mouse ESCs.

2. Materials and methods

The mouse ESC line Royan B1, obtained from the inner cell mass of a C57BL/6 strain mouse blastocyst [26,27], was used in the present study. Royan B1 ESCs were cultured on top of a monolayer of mitomycin-C-treated mouse embryonic fibroblasts (MEF) feeders and at the presence of leukemia inhibitory factor (LIF, Chemicon, ES-GRO, Boronia, Victoria, Australia), as explained previously [26].

To induce differentiation, the ESCs were dissociated from the MEF feeder layer and resuspended in differentiation medium. Then drops (20 μL) of 800 cells were placed on the underside surface of a 100-mm tissue culture dish lids containing Dulbecco’s phosphate buffer saline (PBS, Gibco, USA). After 2 days, the ESCs aggregates in hanging drops called embryoid bodies (EBs) were transferred to suspension culture in 60-mm bacterial dishes (greiner) for an additional 5 days. 7 days old EBs were plated onto 0.1% gelatin-coated 24-well tissue culture plates at a density of one to two EBs per well, and the growth medium was renewed every 2 days. BMP-4 (Sigma, B-2680) was added to culture medium at three final concentrations of 10, 50, and 100 ng/ml throughout the suspension culture. Differentiation medium applied in this study consisted of 0.1 mM β-mercaptoethanol, 1 mM L-glutamine, 1% nonessential amino acid stock and 1% penicillin-streptomycin (all from Gibco except for the first from Sigma) in Knockout™ Dulbecco’s modified eagles medium (DMEM) (high-glucose, with sodium pyruvate formulation; Gibco, USA) supplemented with 15% fetal bovine serum (FBS, ESC qualified, Gibco, USA). With daily observation, the percent of beating EBs was determined up to 30 days after plating, in both control and BMP-4 treated groups.

2.1. Chronotropic responses

For evaluating the function of the ESCs-derived cardiomyocytes, the chronotropic effects of cardioactive drugs including isoprenaline (β1-adrenergic receptor agonist), Bay k8644 (Ca2+ channel activator), phenylephrine (α1-Adrenergic receptor agonist) and carbachol (Muscarinic receptor agonist), (all from Sigma), were assessed at three developmental stages i.e. an early stage (day 7±3), an intermediate stage (day 7±7) and a terminal stage (day 7±14), as previously described by Maltsev et al. [28]. For this, beating frequency rate of 20 individual differentiating cardiomyocyte clusters of the control and 10 ng/ml BMP-4 treated groups was recorded before and 5 min after addition of each
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drug to the medium of the wells, and the changes in pulsation rate were calculated. The final concentration of all drugs applied in this study was $10^{-5}$ M.

2.2. Fluorescent immunostaining

The contracting areas of 5–10 EBs were mechanically dissected at the early (day 7+3), intermediate (day 7+7) and terminal (day 7+14) stages of EBs development. The clusters dispersed using trypsin-EDTA (Gibco, 25300-054). Then, the cells were plated on gelatin-coated 4-well tissue culture plates at a low density and incubated for 60 h. For immunostaining, the cells were fixed using 4% paraformaldehyde, permeated using 0.5% Triton X-100 (Sigma, T-8787), blocked with 10% goat serum (Gibco, 16210-064) and incubated with primary and secondary antibodies for 45 min at 37 °C. Antibodies used in this study included mAbs for cardiac muscle troponin I (CTnI; Chemicon, MAB169) at a dilution of 1:100, mAbs for desmin (Sigma, D1033) at a dilution of 1:20, mAbs for α-actinin at a dilution of 1:800, mAbs for nebulin (Sigma; N-9891) at a dilution of 1:75 and anti-mouse, FITC-conjugated IgG antibody (Sigma, F9006) at a dilution of 1:100. Preparations were examined using fluorescence microscope (Nikon, Japan). Dispersed cells isolated from non-contracting EBs served as negative controls and cells isolated from mouse skeletal muscle served as positive control for nebulin staining.

2.3. Total number of the cells and percent of cardiomyocytes per EB

At day 7+14 of differentiation, the cells of 5 to 6 beating EBs of both control and 10 ng/ml BMP-4 treated groups were singled by trypsin-EDTA. Cell count was carried out using a hemacytometer, and the cells were replated on gelatinized 4-well tissue culture plates and incubated for 48 h. Then, the cells were stained specifically with anti-cardiac troponin I antibody for cardiomyocytes, as well as with propodium iodide (PI, Sigma, P-4170) for all nuclei. Finally, in three different replicates, the ratio of the cardiac troponin I-positive cells per PI-positive nuclei was calculated.

2.4. Transmission electron microscopy

The mechanically dissected contracting areas at day 14 after plating were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h, postfixed in 1% OsO₄ in the same buffer for 1.5 h, dehydrated in graded ethanol, and embedded in Araldite 6005 (Sigma). Ultrathin (50–70 nm) sections were double-stained with uranyl acetate and lead citrate and used for ultrastructural evaluation using a transmission electron microscope (EM900, Zeiss, Germany).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from undifferentiated ESCs and contracting EBs of the early and late developmental stages, in both control and 10 ng/ml BMP-4 treated groups was extracted using RNX plus™ according to the manufacturer’s recommendations (Cinnagen, Tehran, Iran) [29]. 5 μg of total RNA was transcribed into cDNA using oligo-dT, primers and reverse transcriptase (k1632; Fermentas). Primer sets for cardiac α- and β-myosin heavy chain (α-MHC, β-MHC), atrial natriuretic factor (ANF), myosin light chain 2V (MLC-2V), Oct-4 (marker of ESCs) and β-tubuline (as an internal standard) were used in the amplification reactions as described before [30]. The PCR products were size fractionated by 2% agarose gel electrophoresis (fermentas). The transcript for β-tubulin was used for internal normalization, and intensity of
4.2. Total number of the cells and percent of cardiomyocytes per EB

At terminal stage of the EBs’ development, the total number of the cells per EB in the control and BMP-4 treated groups was 93,777.775 (±10,183.499) and 143,999.993 (±16,706.607), and the percentage of the positive-cardiac troponin I cardiomyocytes was 11.33 (±1.40) and 7.286 (±1.28), respectively; both of the differences were significant ($p<0.05$).

4.3. Chronotropic responses

Contracting clusters in both control and BMP-4 treatment groups showed positive or negative chronotropic responses to all administrated drugs, from the early stage (day 7+3) of differentiation. As a whole, cardiac clusters of control group had better responses to cardioactive drugs compared with BMP-4 treated group. But, these differences were only significant at the early stage of differentiation (day 7+3) for Bay, and at the intermediate stage of differentiation (day 7+7) for carbachol (Fig. 2).

4.4. Immunocytochemistry

The cardiomyocytes of the both control and BMP-4 treated groups showed a positive staining for CTnI as well as a weak peripheral staining for desmin, at the early stage (day 7+3) of differentiation, and this staining degree increased at the next developmental stages. Immunostaining with anti-α-actinin mAbs, at the early stage of EBs’ development revealed varying degrees of myofibrillar organization in cardiomyocytes of both control and BMP-4 treated groups; at this stage an early striated pattern was observable. At the intermediate stage of differentiation, an increase in the myofibrillar organization and cross striation was observable in cardiomyocytes of both groups. Finally, at day 14 after plating, a more extent of cross striation was

Fig. 4. Immunostaining of ESCs-derived cardiomyocytes in BMP-4 treated group, at the early, intermediate and late stages of development, respectively presented by A, B, and C: for sarcomeric α-actinin; D, E, F: for desmin; G, H, I: for cardiac-troponin I.
maintaining of Oct-3/4 expression within a certain range is critical for stem cell renewal, with any increase or decrease triggering differentiation to endoderm/mesoderm or trophoectoderm, respectively. From the early to late stage of differentiation, Oct-4 expression increased insignificantly in the control and down-regulated to very low or undetectable levels in BMP-4-treated groups. In fact, fetal bovine serum is containing growth factors, inducing differentiation as well as mitosis/self renewal of ESCs while, BMP-4 treatment causes all cells of the EBs to enter a differentiation pathway instead of remaining undifferentiated. This may be important from the clinical aspect by decreasing the risk of tumorigenesis after transplantation of ESCs or ESC-derived cardiomyocytes, as described previously by Behfar et al. [2] for TGF-β and BMP-2.

In EBs of control group, α-MHC up-regulated and β-MHC down-regulated during early to late stages of development. These changes are consistent with the apparent β- to α-MHC isoform transition during murine cardiac development in vivo [36–39] as well as ESCs differentiation in vitro [34,40]. Some investigators believe that β- to α-MHC isoform shift in rodents is a birth-associated phenomenon related to the change in thyroid hormone status [41–43] that is unlikely to occur during ESCs differentiation [44].

Expression of MLC-2V and ANF is often used to demarcate ventricular-like [10,45–47] and atrial-like [48] cells in ESC cultures, respectively. Although there are some different reports about MLC-2V and ANF expression in vivo, O’Brien et al. [49] regarding the expression of MLC-2V in murine embryo showed that ventricular specification occurs prior to septation and development of distinct atrial and ventricular chambers [10] while, Fijirovandraat [44] believes that MLC-2V is expressed prior to initiation of chamber formation and it is not exclusively restricted to parts that develop into the ventricular chambers. Moreover, ANF is a hormone that in the embryonic mouse heart identifies the forming atrial and ventricular chambers and it is never expressed in nodal tissues. In the adult heart, ANF is only expressed in the atrial appendages but not in the rest of the atria [50–52]. Therefore, in early ESCs-derived cardiomyocytes, ANF expression does not exclusively identify atrial cardiomyocytes [44]. However, in our study, MLC-2V and ANF down-regulated during early to late stage of EBs’ development, in control group. This may be due to chamber specification of MLC-2V and ANF expression or enhancement of non-cardiac cells in the EBs’ outgrowth and the effect of them on total RNA extraction.

In the BMP-4 treated EBs, all of the evaluated cardiac-specific genes down-regulated during early to late stage of EBs’ development, and their expression level was significantly lower than control group. This reduction may be caused by three factors. The first, BMP-4 treatment increased the differentiation of non-cardiac cells, which in turn, can affect the total RNA extracted from whole EBs. The second factor, the decrease in MLC-2V and ANF may be somewhat due to chamber specification. Finally, regarding the similar expression level of cardiac genes in the early stage EBs of control and BMP-4 treated groups, as well as considering the results of immunostaining and ultrastructural analysis of cardiomyocytes of both groups, it can be supposed that the decrease in cardiac genes expression in the late stage EBs may be somewhat due to a latency in development or dedifferentiation of cardiomyocytes. It is clear that differentiation of cell types other than cardiomyocytes in EBs’ outgrowth and effect of them on differentiation of cardiac lineage is noteworthy. Combination of the appropriate cardiac-inducer and non-cardiac-inhibitory growth factors, therefore, may be the best strategy to produce a lot number of cardiomyocytes for clinical applications.

In conclusion, the results demonstrate that BMP-4 treatment in suspension period of EB culture system had an inhibitory effect on cardiomyocyte differentiation from ESCs. BMP-4 treatment decreased the total percent of contracting EBs and reduced the percent of cardiomyocytes per EBs. In contrast, non-cardiac cell differentiation increased with BMP-4 treatment resulting in a dedifferentiation event in cardiomyocytes’ development. Finally, BMP-4 caused a latency or decrease in the chronotropic response of ESCs-derived cardiomyocyte to some cardioactive drugs and disturbance in ultrastructural maturation of them. More efforts, however, is necessary to test the pure effect of BMP-4 treatment on ESCs differentiation to cardiomyocytes in serum-free media as well as the molecular mechanisms involved in these differentiation events.

Acknowledgement

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References

maintaining of Oct-3/4 expression within a certain range is critical for stem cell renewal, with any increase or decrease triggering differentiation to endoderm/mesoderm or trophoectoderm, respectively. From the early to late stage of differentiation, Oct-4 expression increased insignificantly in the control and down-regulated to very low or undetectable levels in BMP-4-treated groups. In fact, fetal bovine serum is containing growth factors, inducing differentiation as well as mitosis/self renewal of ESCs while, BMP-4 treatment causes all of the EBs to enter a differentiation pathway instead of remaining undifferentiated. This may be important from the clinical aspect by decreasing the risk of tumorigenesis after transplantation of ESCs or ESC-derived cardiomyocytes, as described previously by Behfar et al. [2] for TGF-β and BMP-2.

In EBs of control group, α-MHC up-regulated and β-MHC down-regulated during early to late stages of development. These changes are consistent with the apparent β- to α-MHC isoform transition during murine cardiac development in vivo [36–39] as well as ESCs differentiation in vitro [34,40]. Some investigators believe that β- to α-MHC isoform shift in rodents is a birth-associated phenomenon related to the change in thyroid hormone status [41–43] that is unlikely to occur during ESCs differentiation [44].

Expression of MLC-2V and ANF is often used to demarcate ventricular-like [10,45–47] and atrial-like [48] cells in ESC cultures, respectively. Although there are some different reports about MLC-2V and ANF expression in vivo, O’Brien et al. [49] regarding the expression of MLC-2V in murine embryo showed that ventricular specification occurs prior to septation and development of distinct atrial and ventricular chambers [10] while, Fijirovandraat [44] believes that MLC-2V is expressed prior to initiation of chamber formation and it is not exclusively restricted to parts that develop into the ventricular chambers. Moreover, ANF is a hormone that in the embryonic mouse heart identifies the forming atrial and ventricular chambers and it is never expressed in nodal tissues. In the adult heart, ANF is only expressed in the atrial appendages but not in the rest of the atria [50–52]. Therefore, in early ESCs-derived cardiomyocytes, ANF expression does not exclusively identify atrial cardiomyocytes [44]. However, in our study, MLC-2V and ANF down-regulated during early to late stage of EBs’ development, in control group. This may be due to chamber specification of MLC-2V and ANF expression or enhancement of non-cardiac cells in the EBs’ outgrowth and the effect of them on total RNA extraction.

In the BMP-4 treated EBs, all of the evaluated cardiac-specific genes down-regulated during early to late stage of EBs’ development, and their expression level was significantly lower than control group. This reduction may be caused by three factors. The first, BMP-4 treatment increased the differentiation of non-cardiac cells, which in turn, can affect the total RNA extracted from whole EBs. The second factor, the decrease in MLC-2V and ANF may be somewhat due to chamber specification. Finally, regarding the similar expression level of cardiac genes in the early stage EBs of control and BMP-4 treated groups, as well as considering the results of immunostaining and ultrastructural analysis of cardiomyocytes of both groups, it can be supposed that the decrease in cardiac genes expression in the late stage EBs may be somewhat due to a latency in development or dedifferentiation of cardiomyocytes. It is clear that differentiation of cell types other than cardiomyocytes in EBs’ outgrowth and effect of them on differentiation of cardiac lineage is noteworthy. Combination of the appropriate cardiac-inducer and non-cardiac-inhibitory growth factors, therefore, may be the best strategy to produce a lot number of cardiomyocytes for clinical applications.

In conclusion, the results demonstrate that BMP-4 treatment in suspension period of EB culture system had an inhibitory effect on cardiomyocyte differentiation from ESCs. BMP-4 treatment decreased the total percent of contracting EBs and reduced the percent of cardiomyocytes per EBs. In contrast, non-cardiac cell differentiation increased with BMP-4 treatment resulting in a dedifferentiation event in cardiomyocytes’ development. Finally, BMP-4 caused a latency or decrease in the chronotropic response of ESCs-derived cardiomyocyte to some cardioactive drugs and disturbance in ultrastructural maturation of them. More efforts, however, is necessary to test the pure effect of BMP-4 treatment on ESCs differentiation to cardiomyocytes in serum-free media as well as the molecular mechanisms involved in these differentiation events.

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References


Use of Long-term Cultured Embryoid Bodies May Enhance Cardiomyocyte Differentiation by BMP2

Yoon Young Kim,¹ Seung-Yup Ku,¹,² Jiho Jang,¹ Sun Kyung Oh,¹,² Hee Sun Kim,¹,² Seok Hyun Kim,¹,² Young Min Choi,¹,² and Shin Yong Moon¹,²

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Purpose: Human embryonic stem cells (hESCs) can proliferate for a prolonged period and differentiate into cardiomyocytes in vitro. Recent studies used bone morphogenetic protein 2 (BMP2) to generate cardiomyocytes from hESCs, however, all those studies used early embryoid bodies (EBs) and did not retrieve cardiomyocytes with a high yield. In this study, we treated long-term cultured EBs with BMP2 in order to promote differentiation into cardiomyocytes from hESCs.

Materials and Methods: hESC lines, including SNUhES3 and SNUhES4, were used in this study. Undifferentiated hESC colonies were detached to form EBs and cultured for up to 30 days. These long-term cultured EBs were differentiated into cardiomyocytes in serum-containing media. In our protocol, BMP2 was applied for 5 days after attachment of EBs. Cardiac specific markers, beating of differentiated cells and electron microscopic (EM) ultrastructures were evaluated and analyzed.

Results: Compared to 10-day or 20-day EBs, 30-day EBs showed a higher expression level of cardiac specific markers, Nkx2.5 and α-myosin heavy chain (αMHC). Treatment of BMP2 increased expression of cardiac troponin (cTn) I and α-actinin when evaluated at 20 days after attachment of 30-day EBs. Beating of differentiated cells was observed from 7 to 20 days after attachment. Moreover, EM findings demonstrated fine structures such as Z bands in these differentiated cardiomyocytes. These long-term cultured EBs yielded cardiomyocytes with an efficiency of as high as 73.6% when assessed by FACS.

Conclusion: We demonstrated that the use of long-term cultured EBs may enhance differentiation into cardiomyocytes from hESCs when treated with BMP2.

Key Words: Bone morphogenetic protein 2, cardiomyocytes, cell differentiation, embryoid bodies, embryonic stem cells, long-term

INTRODUCTION

Human embryonic stem cell, derived from inner cell mass (ICM) of preimplantation embryos, can proliferate for a prolonged period in vitro and can differentiate into various cell types under suitable environment. Therefore, hESCs are considered as a candidate cell source of cell-based therapies for heart diseases. Since 2001, there have been numerous studies of hESC-derived cardiomyocytes, and these studies used spontaneous differentiation, low-serum culture condition and co-culture with endoderm-like cells. However, practical methods using specific signaling molecules that are known to be efficient in differentiation into cardiomyocytes from hESCs are still limited.

Development of uncommitted mesodermal precardiac cells to early cardiomyocytes is regulated by stimulatory signals that are secreted from anterior primitive endoderm. Bone morphogenetic proteins (BMPs) signaling is main signaling pathway regulating the cardiomyogenesis. Among BMPs, BMP2 is known to play a crucial role in the induction of heart formation of vertebrate embryos.

In hESCs, BMP2 is known to be an inducer for mesoderm or cardiac differentiation. Tomescot et al. showed that BMP2 treatment turned on
Use of Long-term Cultured Embryoid Bodies May Enhance Cardiomyocyte Differentiation by BMP2

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INTRODUCTION

Human embryonic stem cells, derived from inner cell mass (ICM) of preimplantation embryos, can proliferate for a prolonged period in vitro1,2 and can differentiate into various cell types under suitable environment. Therefore, hESCs are considered as a candidate cell source of cell-based therapies for heart diseases. Since 2001, there have been numerous studies of hESC-derived cardiomyocytes,3-10 and these studies used spontaneous differentiation,3,4,6,7,10 low-serum culture condition8,9 and co-culture with endoderm-like cells.5 However, practical methods using specific signaling molecules that are known to be efficient in differentiation into cardiomyocytes from hESCs are still limited.

Development of uncommitted mesodermal precardiac cells to early cardiomyocytes is regulated by stimulatory signals that are secreted from anterior primitive endoderm.11 Bone morphogenetic proteins (BMPs) signaling is main signaling pathway regulating the cardiomyogenesis. Among BMPs, BMP2 is known to play a crucial role in the induction of heart formation of vertebrate embryos.12,13

In hESCs, BMP2 is known to be as an inducer for mesoderm or cardiac differentiation. Tomescot et al. showed that BMP2 treatment turned on
after plating of EBs. Clusters showed regularity in beating and the beating lasted more than 30 days in vitro (Fig. 4A-d and h, Supplementary Fig. 1). Expressions of cardiac specific markers were significantly increased by treatment with BMP2. Immunostaining demonstrated the expressions of transcription factor Nkx2.5, structural protein α-MHC (Fig. 4B, upper panel) and cardiac specific
Q-PCR analysis showed that the expression of Nkx2.5 and aMHC was increased in BMP2-treated group more than untreated group. Especially, BMP2 treatment strikingly increased the expression of structural protein aMHC (Fig. 4D).

Ultrastructures of hESC-derived cardiomyocytes were observed using transmission electron microscopy. Differentiated cells were analyzed to confirm the possession of Z bands. Z bodies, which are precursors of Z bands, and unorganized sarcomeric structure were abundant in differentiated cells (Fig. 4E-a). Differentiated cells from BMP2-treated group showed many unorganized (Fig. 4E-b) as well as organized (Fig. 4E-c and d) Z bands. This ultrastructural analysis showed various stages of Z band development even in a single cell, suggesting that high proportion of cells was in progress for differentiation.

**Yield of cardiomyocytes differentiated from long-term cultured EBs**

To find out the efficiency, the proportion of cardiac specific marker-positive cells was evaluated by FACS analysis. Proportion of cardiac lineage specific cells was significantly higher than those in untreated differentiated cells (data not shown). As high as 73.6% of cells in BMP2-treated group (range, 10.8 - 73.6%) were doubly positive for Nkx2.5 and aMHC (Fig. 5). These results clearly show that BMP2 could be an effective inducing factor for differentiation into cardiomyocytes, and the use of long-term cultured EBs may enhance the yield.

**DISCUSSION**

To our best knowledge, this is the first study that used long-term cultured EBs in differentiation into cardiomyocytes from hESCs. When these long-term cultured EBs differentiated by treatment with BMP2, their differentiation efficiency was as high as 73.6%. This figure is higher than that in previous reports.\(^1\) This high yield of differentiation demonstrated the use of long-term cultured EBs with BMP2 treatment may enhance differentiation into cardiomyocytes in the cell lines used. In addition, our protocol generated healthier, vigorous, contracting clusters, and the beating lasted more than 30 days in vitro. We also demonstrated that differentiated cells from long-term cultured EBs with BMP2 possessed many unorganized and organized Z bands. These findings suggest that differentiation was ongoing from precursors of Z bands to organized mature forms.

To date, many methods have been utilized for generation of cardiomyocytes from hESCs, such as spontaneous differentiation,\(^3,4,6,7\) co-culture with END-2 cell line\(^5,8,9\) and serum-free or low-serum culture condition.\(^8-10\) As for cardiac lineage inducing agents, 5'-aza-deoxycytidine and BMP4 were among a few molecules that are known to work in differentiation into cardiomyocytes from hESCs.\(^4\) BMPs are well-known morphogen that contribute to differentiation into cardiac lineage; e.g., BMP signaling is crucial in mesodermal induction and cardiac formation in vertebrate.\(^14\) Tomescot et al. used BMP2 to induce differentiation into cardiac committed cells that were used in a rat infarct model,\(^15\) and Pal et al. showed that...


Effects of oxytocin on cardiomyocyte differentiation from mouse embryonic stem cells

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Abstract

This study sought to investigate the presence of oxytocin receptors and the possible biological role of oxytocin as an effective factor in the differentiation of embryonic stem cells (ESCs) into cardiomyocytes. Mouse ESCs were cultivated in hanging drops to form embryoid bodies (EBs). The EBs were then treated with and without oxytocin (experimental and control groups). Up to 30 days after plating, contraction and beating frequency were monitored and evaluated daily. The growth characteristics of the ESC-derived cardiomyocytes were assessed by cardioactive drugs, immunocytochemistry, transmission electron microscopy (TEM) and reverse transcription-polymerase chain reaction (RT-PCR). In the experimental group, the percentage of the EBs with spontaneous contraction was significantly increased from 17th day onward. The spontaneous beating frequency of each EB in both groups was also changed with cardioactive drugs such as Bay K, carbachol, isoprenaline and phenylephrine. However, in the experimental group, changes with isoprenaline were more pronounced at the early and intermediate stages of cardiomyocyte development. The beating cells of both groups, stained positive with anti-α-actinin, desmin, cardiac troponin I and connexin antibodies, and revealed similar ultrastructural features. Oxytocin receptors were detected on the ESCs and derived-differentiated cells. In addition, cardiac-specific genes such as cardiac α- and β-myosin heavy chain, myosin light chain-2v, and atrial natriuretic factor were also detected in the ESC-derived differentiated cells of both groups. In the experimental group, all the specific genes, with the exception of α-myosin heavy chain, were more pronounced at the early stage of cardiomyocyte development. In conclusion, oxytocin has receptors on undifferentiated ESCs and derived differentiated cells, and in spite of better improvement of the EBs with spontaneous contraction, it can only promote the early maturation of ESC-derived cardiomyocytes in terms of chronotropic responses and expression of cardiac-specific genes, and have no effect on ultrastructural characteristics of cardiomyocytes in any stage of development.

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Keywords: Mouse embryonic stem cells; Cardiomyocyte differentiation; Chronotropic response; Oxytocin; Oxytocin receptors

1. Introduction

Oxytocin, a nonapeptide hormone, is a very abundant neuropeptide secreted mainly from the hypothalamic nuclei. However, oxytocin is also produced in peripheral tissues, e.g. uterus, placenta, amnion, corpus luteum, testis and heart [1]. Oxytocin and oxytocin receptors have long been recognized to be mainly involved in the regulation of reproductive functions, such as the induction of uterine contractions, milk ejection reflex and ovulation [1]. Many other effects of oxytocin have been found in the regulation of different behavioral and neuro-mediated functions, including sexual and maternal behavior, memory, cognition, tolerance, adaptation, food and drink intake, modulation of anorexia and cardiovascular functions [2]. New functions have recently been reported for oxytocin as a positive and negative regulator of cell proliferation in different tissues [3] and a differentiation factor for myoepithelial cells in the mouse mammary gland [4].
increased in each EB as compared to that in each EB in the control group (10.4% vs. 7.1% at the early stage and 11.2% vs. 9.2% at the late stage, respectively, \( p < 0.05 \)).

3.2. Pharmacological responses of ESC-derived cardiomyocytes

The chronotropic effects of cardioactive drugs on the in vitro function of ESC-derived cardiomyocytes in the control and experimental groups at three developmental stages are summarized in Fig. 3, B–D. The increase in beating frequency by Bay K as a Ca\(^{2+}\)-channel activator was alike in both control and experimental groups and that no significant difference was found between these groups at all the developmental stages.

Adrenoceptors functioning was determined by treating the contracting cells with isoprenaline as a \( \beta \)-1-adrenoceptor agonist and phenylephrine as a \( \alpha \)-1-adrenoceptor agonist. The rate of beating was subsequently monitored. Significant positive chronotropic effects on the cardiomyocytes were observed after the application of isoprenaline from an early stage in both groups. At the early and intermediate stages, the increase in beating frequency of the experimental group was more than that of the control group, the difference being significant (\( p < 0.05 \)). However, the response to this drug was similar at the late stage.

Phenylephrine enhanced the rate of beating frequency at all the developmental stages in both groups. The response to this drug was the same between both groups at all the developmental stages.
Evaluation of Mouse Embryonic Stem Cells Plasticity Through Different Differentiation Protocols

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Abstract. The present study was designed to evaluate the plasticity of mouse embryonic stem cells (ESCs). Cardiac and neural differentiation of the mouse ESCs was initiated by embryoid bodies (EBs) formation in hanging drops and in suspension. After aggregation the were transfered to the suspension culture and then plating on to gelatin-coated tissue culture plates. Retinoic acid was added to culture medium through out the suspension period. Cultures were observed daily with an inverted microscope for the appearance of contracting clusters and neural filaments. At the early, intermediate and terminal stages of differentiation, the choronotropic responses of cardiomyocytes to cardioactive drugs were assessed, and the cardiomyocytes immunostained for citokeratin, titin and betaIIItubulin. Our data suggest that ESCs can differentiate into functional mature cardiomyocytes in vitro. Furthermore, ESC-cardiomyocytes may provide an ideal model for the study of cardiomyocytic development and may be useful for cell therapy of various cardiac diseases.

Keywords: embryonic stem cells, mouse, plasticity, differentiation

Embryonic stem cells (ESCs) are expected to become a powerful tool for future regenerative medicine and developmental biology due to their capacity for self-renewal and pluripotency (12). During the last decade, embryonic stem cells (ESC) have unleashed new avenues in the field of developmental biology and emerged as a potential tool to understand the molecular mechanisms taking place during the process of differentiation from the embryonic stage to adult phenotype. Developmental fate of differentiating stem cells depends on the complex ‘cocktail’ of growth factors, signaling molecules, and extracellular matrix (ECM) proteins constituting the developmental ‘niche’ in which the cells exist (4,5). In general, they share the following characteristics: (1) a high capacity for self-renewal; (2) the potential for multipotent differentiation potential; (3) the ability to be cultured ex vivo and used for tissue engineering (reprogramming); and (4) plasticity (transdifferentiating ability) (1,2,4,10, 11,14). These unique properties have provided added impetus to this area of research. Among the specialized cell types that can be induced in vitro, cardiomyocytes are particularly conspicuous, giving rise to rhythmically contracting structures containing cells highly reminiscent of normal human heart cells (8). Myocardial infarction (MI) and congestive heart failure are the leading causes of morbidity and mortality worldwide (3). Owing to the paucity of donor hearts for transplantation, and existing pharmacological approaches being insufficient
to reverse progressive heart failure, establishment of alternative methods has emerged at an astonishing rate. Cell replacement therapy would consider transplantation of appropriate heart cells to the infarcted area, regenerating at least part of the dysfunctional myocardium, securing fresh heart muscle necessary to regain organ function.

Theoretically, ESC-derived cardiomyocytes are apt for the purpose of cardiac cell therapy since they demonstrate reproducible differentiation of multiple cardiac cell types in large numbers, prolonged survival and site-specific engraftment which are the keys to success in tissue replacement therapy (5,12).

The aim of this study was to evaluate the plasticity of mouse embryonic stem cells in vitro. To assess the ability of mouse embryonic stem cells were chosen different differentiation protocols.

MATERIALS AND METHODS

We used at our experiment the mouse ES cell line CDE1 with a normal karyotype, at 10th passages, obtained from inner cell mass of a CD1/EGFP strain mouse blastocysts in our laboratory. CDE1 cells were kept on primary embryonic mouse fibroblast feeder layer, in Dulbecco’s modified Eagle’s medium (KO-DMEM medium) (GIBCO) supplemented with glutamax (Gibco, 100x), 50 µg/ml streptomycin (SIGMA), 50U/ml penicillin (SIGMA), 50mM β-mercaptoethanol (ME) (SIGMA), 0.1mM non-essential amino acids (GIBCO), 1000 units/ml of leukemia inhibitory factor (ESGRO) and 20% fetal calf serum (FCS) (HyClone).

Two days before the differentiation, ES cells were passaged into gelatin (0.1%) (SIGMA) coated petri dishes (Greiner) in the ES culture medium.

On the appointed day 0 of differentiation, we passage the cells into 10 cm bacteriological dishes, containing 5x10^6 ES cells in 10 ml differentiation medium (Day 0 of differentiation). As differentiation medium the IMDM (Gibco) medium supplemented with 0.6 m/m% penicillin, 1 m/m% streptomycin and 20v/v% FCS was employed. MTG (monothyoglycerol) 3 l/ml was always freshly added to the differentiation medium. For hanging drop production 2x10^5 cells/ml containing cell-suspension was prepared in IMDM differentiation medium.

From this suspension, three Petri dish covers with 70 hanging-drops (a drop of 20 µl, contained 400 mouse ES cells) and one suspension Petri dish (3 ml cell suspension) were set in culture for two days (Day 2). Cell aggregates were obtained after 5–6 hrs, while EBs were morphologically completed after 2 days, by each procedure. The second step was to plate the EBs on a gelatin-coated surface for 17 days (Day2+7). Best EBs from the hanging-drops and from the cell suspension were separately harvested and pooled EBs from each variant were further grown on 24-well tissue culture dishes. Prior cultivation of EBs, a rounded shaped histologic cover slip was introduced within each well in order to later perform immuno-staining. All culture dishes so prepared, were gelatin-coated before addition of EBs.

For cardiomyocytes differentiation, 48 EBs were further plated in two 24-well tissue culture plates within IMDM medium/MTG. Every second day the medium was changed for 17 days. For neuronal differentiation, 48 EBs were further plated in two 24-well tissue culture plates within IMDM medium/MTG and 10^{-6}M retinoic acid (RA). Every second day the medium was changed for 17 days, but RA was added at the first four days.

With daily observation, the percent of beating EBs was determined up to 19 days after plating. For evaluating the function of the ESCs derived cardiomyocytes, the chronotropic effects of cardioactive drugs including isoprenaline, phenyleprine and carbacol were assessed at three developmental stages, an early stage an intermediary stage and a terminal stage.
Was clearly seen under the RA treatment and best results were obtained from the EBs produced in suspension. The neuronal inductive influence of RA treated EBs was earlier reported by Stübing (13). Committed neuronal lineage were observed in EBs from the suspension variant 12 days after plating (day2+12). Specific morphology of neuronal cells and the networks of synaptically coupled cells are clearly seen by immunocytochemical detection of betaIII tubulin (Fig.1) and cytokeratin Endo-A (Fig.1). However, within EBs there were present several undifferentiated, pluripotent cells expressing the Oct-4 marker.

Contracting clusters showed positive or negative chronotropic responses to all administrated drugs, from the early stage (day 7 + 3) of differentiation.

The increase in beating frequency by β1-adrenoceptor agonist, Isoprenaline was alike in both control and experimental groups. The rate of beating was, subsequently, monitored. Significant positive chronotropic effects on the cardiomyocytes were observed after the application of isoprenaline from an early stage. However, the response to this drug was similar at the late stage. Phenylephrine enhanced the rate of beating frequency at all the developmental stages.
Isolation of Mouse Marrow Mesenchymal Progenitors by a Novel and Reliable Method

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Key Words. Mesenchymal progenitor cells · Mixed leukocyte reaction · Immune regulation

ABSTRACT

Bone marrow contains a population of rare progenitor cells capable of differentiating into osteoblasts, chondrocytes, adipocytes, myoblasts, and hematopoiesis-supporting stromal cells. These cells, referred to as mesenchymal progenitor cells (MPCs), can be purified and culture-expanded from animals and humans. Using bone-marrow-conditioned medium combined with basic fibroblast growth factor, we cultured a relatively homogeneous population of MPCs from murine bone marrow, which uniformly expressed stem cell antigen-1, CD29, CD44, c-kit, and CD105, while being negative for expression of CD45, CD31, and CD34. In vitro differentiation assays showed the tripotential differentiation capacities of these cells toward adipogenic, osteogenic, and chondrogenic lineages. Most importantly, immunophenotypic analyses demonstrated that MPCs did not express major histocompatibility complex class II molecules or the T-cell costimulatory molecules CD80 and CD86, consistent with further investigation showing that MPCs failed to elicit a proliferative response from allogeneic lymphocytes. Moreover, when allogeneic or third-party MPCs were added to T cells stimulated by allogeneic lymphocytes or the potent T-cell mitogen concanavalin-A, a significant reduction in T-cell proliferation was observed. In conclusion, our data demonstrate that we successfully isolated and culture-expanded a relatively homogeneous population of MPCs from adult murine bone marrow. Additionally, these primary cells could suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. This immunoregulatory feature of MPCs strongly implies that they may have potential applications in allograft transplantation. Stem Cells 2003;21:527-535

INTRODUCTION

In addition to hematopoietic stem cells, bone marrow contains stem/precursor cells for several mesenchymal cell types, such as osteoblasts, chondrocytes, adipocytes, and myoblasts [1]. These mesenchymal progenitor cells (MPCs) have also been referred to by diverse, yet indistinct, denominations such as colony-forming fibroblastic cells [2], stromal fibroblasts, marrow stromal stem cells [3], mesenchymal stem cells [4], and marrow stromal cells [5].

In recent studies, MPCs from humans and baboons have been shown to exhibit low immunogenicity and demonstrate significant suppressive activity in cell cultures containing alloreactive T cells [6, 7]. Immunologically, MPCs express surface markers including vascular cell...
and then subcultured into culture flasks. Cells resulting from this replating were designated first-passage cells. At each passage, cells were typically diluted 1:2. After the fourth passage, cells were cultured in α-MEM complete medium supplemented with 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech; Rocky Hill, NJ; http://www.peprotech.com). At about the eighth passage, cells were harvested for the experiments described below.

**Flow Cytometry Analysis**

Trypsinized cells (2 × 10^6) were washed with fluorescence-activated cell sorting (FACS) buffer (2% FBS, 0.1% NaOH in PBS), incubated on ice for 30 minutes, and stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse stem cell antigen (Sca)-1 (eBioscience; San Diego, CA; http://www.ebioscience.com), CD45, CD34 (Becton Dickinson; Sun, Guo, Xiao et al. 529

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from MPCs cultured in control or osteoinductive medium for 9 days using Trizol (GIBCO/BRL; Grand Island, NY; http://www.invitrogen.com) according to the manufacturer’s instructions and then treated with DNase (Promega; Madison, WI; http://www.promega.com). RT-PCR was performed by using the mRNA selective PCR kit (Takara Shuzo; Kyoto, Japan; http://www.takara.co.jp). A tenfold dilution of each sample was PCR amplified to achieve signals within the linear amplification range. The primers used were as follows: hypoxanthine phosphoribosyl-transferase, 5′-GCTGGTGAAAGGACCTCT-3′ and 5′-CACAGGACTA GAAACCTGC-3′ [27]; osteocalcin (OCN), 5′-TGTGACA AAGCCTTCATGTCC-3′ and 5′-AAATGGTATACCGTA GATGCG-3′; and osteopontin (OPN), 5′-ACACTTTCAC TCAATCGTCC-3′ and 5′-TTGTCCCTTGTGTTGCC-3′ [28]. Cycles for each primer pair were empirically determined so as to yield a product within the early exponential phase of synthesis to assure comparative analyses. Amplified DNA fragments were separated on a 2% agarose gel containing 0.1 µg/ml ethidium bromide and visualized under UV light.

**Histochemical and Cytological Staining**

**Alkaline Phosphatase Assay**

The BCIP/NBT kit (Zhongshan Company; Beijing, China; http://www.zsbio.com) was used to assess ALP activity according to the manufacturer’s instructions.

**von Kossa Staining**

Mineralization of ECM was visualized by von Kossa staining. Briefly, cells incubated in osteogenic medium for 4 weeks were fixed with 4% paraformaldehyde for 60 minutes at room temperature. After being rinsed with distilled water, cells were overlaid with 1% silver nitrate solution in the absence of light for 30 minutes. Then, cells were washed several times with distilled water and developed under UV light for 60 minutes. Excess silver staining was removed by washing several times with 5% sodium hyposulfite solution.

**Oil Red-O Staining**

Cells were fixed for 60 minutes at room temperature in 4% formaldehyde/1% calcium, washed with 70% ethanol,
REVIEW

Embryonic stem cell differentiation: emergence of a new era in biology and medicine

Gordon Keller
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The discovery of mouse embryonic stem (ES) cells more than 20 years ago represented a major advance in biology and experimental medicine, as it enabled the routine manipulation of the mouse genome. Along with the capacity to induce genetic modifications, ES cells provided the basis for establishing an in vitro model of early mammalian development and represented a putative new source of differentiated cell types for cell replacement therapy. While ES cells have been used extensively for creating mouse mutants for more than a decade, their application as a model for developmental biology has been limited and their use in cell replacement therapy remains a goal for many in the field. Recent advances in our understanding of ES cell differentiation, detailed in this review, have provided new insights essential for establishing ES cell-based developmental models and for the generation of clinically relevant populations for cell therapy.

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos (Evans and Kaufman 1981; Martin 1981). Their importance to modern biology and medicine derives from two unique characteristics that distinguish them from all other organ-specific stem cells identified to date. First, they can be maintained and expanded as pure populations of undifferentiated cells for extended periods of time, possibly indefinitely, in culture. Unlike transformed tumor cell lines, ES cells can retain normal karyotypes following extensive passaging in culture. Second, they are pluripotent, possessing the capacity to generate every cell type in the body. The pluripotent nature of mouse ES cells was formally demonstrated by their ability to contribute to all tissues of adult mice, including the germline, following their injection into host blastocysts (Bradley et al. 1984). In addition to their developmental potential in vivo, ES cells display a remarkable capacity to form differentiated cell types in culture (Keller 1995; Smith 2001). Studies during the past 20 years have led to the development of appropriate culture conditions and protocols for the generation of a broad spectrum of lineages. The ability to derive multiple lineages from ES cells opens exciting new opportunities to model embryonic development in vitro for studying the events regulating the earliest stages of lineage induction and specification. Comparable studies are difficult in the mouse embryo and impossible in the human embryo. In addition to providing a model of early development, the ES cell differentiation system is viewed by many as a novel and unlimited source of cells and tissues for transplantation for the treatment of a broad spectrum of diseases. The isolation of human ES cells (hES) in 1998 dramatically elevated the interest in the cell therapy aspect of ES cells and moved this concept one step closer to reality (Thomson et al. 1998). This review details the current status of mouse and human ES cell differentiation from both the developmental biology and cell replacement perspectives. The first sections of the review highlight successes to date in the generation and characterization of mature populations, while the final section outlines the challenges for the future with a focus on the identification of progenitor cells representing the earliest stages of embryonic lineage development. The reader is referred to other recent reviews that provide additional details for many of the subjects covered here (Kyba and Daley 2003; Nir et al. 2003; Hornstein and Benvenisty 2004; Lang et al. 2004; Pera and Trounson 2004; Rippon and Bishop 2004; West and Daley 2004). For the purpose of this review, the term ES will be used in reference to mouse cells and hES for human cells.

Maintaining undifferentiated ES cells

ES cells were initially established and maintained by co-culture with mouse embryonic feeder cells (Evans and Kaufman 1981; Martin 1981). Subsequent studies identified leukemia inhibitory factor (LIF) as one of the feeder-cell-derived molecules that plays a pivotal role in the maintenance of these cells [Smith et al. 1988; Williams et al. 1988; Stewart et al. 1992]. In the presence of appropriate batches of fetal calf serum (FCS), recombinant LIF can replace the feeder cell function and sup-

Keywords: ES cells; differentiation; mesoderm; endoderm; ectoderm; embryonic development

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Lineage development from ES cells


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Establishment of an embryonic stem cell line from blastocyst stage mouse embryos

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2)“Prof. dr. Ioan Chiricuţă” Oncological Institute, Cluj-Napoca, Romania
3)Genetic Modification Program Group, Agricultural Biotechnology Center, Godollo, Hungary

Abstract
Embryonic stem cells have the ability to remain undifferentiated and proliferate in vitro while maintaining the potential to differentiate into derivatives of all three embryonic germ layers. The aim of the present study was to establish mouse ES lines from blastocyst stage embryos obtained after CD1/EGFP mice superovulation. We isolated, cultured and determined the characteristics of mouse embryonic stem cells in early passages, which were first described by Evans M and Kaufman M. Therefore, we evaluated the morphological criteria for the approval of ES cells in early expansion stage. Two cell lines were isolated (CDE1 and CDE2) and analyzed. They showed similar characteristics to those reported earlier for blastocyst-derived ES cell lines.

Keywords: mouse embryonic stem cells, embryonic stem cell line, immunostaining, embryoid bodies.

Introduction
Embryonic stem cells have the ability to remain undifferentiated and proliferate in vitro while maintaining the potential to differentiate into derivatives of all three embryonic germ layers [1]. Pluripotent embryonic stem cells (ES) are harvested from the inner cellular mass (ICM) of the pre-implantation embryos on E3.5 and cultured thereafter in vitro [2, 3]. The stem cells bear two main properties: unlimited self-renewal in an undifferentiated state without senescence and pluripotency. The ES cells may differentiate into any cell type of the three embryonic layers: endoderm, ectoderm and mesoderm [2, 4].

The interest on mouse ES cells has increased after the isolation of teratocarcinomas [5]. These are gonad tumors made of a mixture of differentiated cells belonging to all three germinal layers (endoderm, ectoderm and mesoderm) and of undifferentiated cells similar to the early embryonic cells named embryonic carcinomas (EC) [6]. The carcinomas cell lines (EC) were the first pluripotent established cell lines to be generated. The EC may be cultured and maintained in vitro as such or may be induced to differentiate into any of the three germ layer cells [2, 7]. The ICM cells proliferate indefinitely in vivo in an undifferentiated state, before becoming progressively committed to give rise to specific cells [8, 9].

Although the pluripotent ES cells have a limited lifetime within the embryo, they could be indefinitely grown within appropriate in vitro conditions. To proliferate in such an undifferentiated state the cells need either leukemia inhibitory factor (LIF) in their culture medium, or to be grown on a feeder layer of inactivated mouse embryonic fibroblasts (MEF) [10, 11].

The aim of this paper was to isolate embryonic stem cells from mouse blastocysts and to create and characterize a stable ESCs line for future studies.

Materials and Methods
Mice, embryos and isolation of ES cells
Embryos were collected from 5 to 8-week-old female mice. A total of 10 mice from CD1/EGFP stain (Agricultural Biotechnology Centre, In vivo Gene Expression and Regulation Group, Godollo) were used. The mice were housed at 25°C under 50–60% relative humidity with a 12 hours light / 12 hours dark photo-period (lights on at 06:00 hr.) until they were used in the experiments. Mice were fed with commercial pelleted food and water ad libitum.

Experimental female mice were grown in standard mobile mouse cages equipped with watering system (Thoren cages system) were kept under 12 hours light and 12 hours dark cycle. Superovulation was induced by injecting 7 units of pregnant mare’s serum gonadotropin (PMSG-Folligon, Intervet) i.p. followed by 7 units of human chorionic gonadotropin (hCG-Chorulon, Intervet) i.p. within a 48 hours interval. After the hCG injection, the females were immediately placed in cages with males (1:1) in order to copulate and 29.5 hours thereafter they were examined for the presence of the vaginal plug. The sign signifies mating and its presence sets the first day of pregnancy (d1).

Blastocysts were collected in the morning of day 3.
Establishment of an embryonic stem cell line from blastocyst stage mouse embryos

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DIFFERENTIATION OF PLURIPOTENT MOUSE EMBRYONIC STEM CELLS LINE

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Summary

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocysts. They are pluripotent cells that have the ability to differentiate into different cell lines. This study aims to demonstrate the differentiation potential of mouse embryonic stem cells (ESCs). As the first stage of differentiation ESCs were transferred to gelatin-coated plates, and after 24 hours cell aggregation were performed in hanging drops. After organization of embryoid bodies (EBs) were transferred in to gelatin-coated plates. Cultures were observed daily with an inverted microscope for the appearance of contracting clusters and neural filaments. Our data show the ability of pluripotent stem cells line, important sources for regenerative medicine.

Key words: mouse, embryonic stem cells, pluripotent, differentiation

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Introduction

The generation of various differentiated cells from pluripotent embryonic stem (ESCs) cells, which is a renewable resource, provides insights for studying not only the mechanism of early development in vitro but also cell transplantation therapy (Anversa P., et al. 1991, Hajime S., et al. 2006).

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 (Evans and Kaufman, 1981; Thomson et al., 1998, Aejaz et al. 2007) 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 (Conley et al., 2005).

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 (Thomson et al., 1998; Wobus, 2001; Draper et al., 2004). 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, endoderm, and ectoderm (Keller, 2005).

The formation and early differentiation of embryoid bodies (EBs) as a principal step in the differentiation of ESCs in vitro. ESCs cells cultivated as embryo-like aggregates, called embryoid bodies (EBs), differentiate in vitro into cellular derivatives of all three primary germ layers of endodermal, ectodermal, and mesodermal origin. ES cell lines develop from an undifferentiated stage resembling cells of the early embryo into terminally differentiated stages of the cardiogenic, myogenic, neurogenic, hematopoietic,
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adipogenic, or chondrogenic lineage, as well as into epithelial, endothelial, and vascular smooth muscle (VSM) cells (Hesler et al., 1997). Terminally differentiated ES cells also show pharmacological and physiological properties of specialised cells: in vitro differentiated cardiomyocytes have characteristics typical of atrial-, ventricular-, Purkinje-, and pacemaker-like cells, and neuronal cells are characterized by inhibitory and excitatory synapses. Neuronal, cardiac, and VSM cells express functional receptors typical for each cell type. Differentiation of ES cells in EBs provides a suitable model not only to understand the process of early embryonic development but also to identify molecules involved in the regulation of the differentiation processes (Wobus et al., 2005).

The aim of this study was to evaluate the differentiation potential of mouse embryonic stem cells in vitro.

**Methods**

**Culture of undifferentiated mouse ES cells**

Embryonic stem (ES) cells of line R1 (with a normal karyotype, at 18th passages) were cultivated on feeder layer of primary mouse embryonic fibroblasts on gelatin (0.1%)-coated petri dishes in DMEM (Gibco) supplemented with 15% heat-inactivated fetal calf serum (FCS) (Hyclone), L-glutamine (2 mM, Sigma), non-essential amino acids (Sigma), penicillin–streptomycin (Gibco), β-mercaptoethanol (Sigma, final concentration 0.1 mM), and 10 ng/ml LIF (Esgro).

**Differentiation of mouse ES cells**

Two days before the differentiation, ES cells were passaged into gelatin (0.1%) (Sigma) coated petri dishes (Greiner) in the ES culture medium. For cardiac and neuronal differentiation, R1 cells (n=400) were used to form embryoid bodies (EBs) that were cultivated in hanging drops with Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 10% FCS, 450 µM α-monothioglycerol (MTG, Sigma), L-glutamine, penicillin–streptomycin. MTG (monothioglycerol) 3 μl/ml was always freshly added to the differentiation medium. Cell aggregates were obtained after 5–6 hrs, while EBs were morphologically completed after 2 days. At day 2, EBs were transferred into bacteriological petri dishes and cultured in suspension. Prior cultivation of EBs, a rounded shaped histologic cover slip was introduced within each well in order to later perform immuno-staining. Retinoic acid (RA, Sigma, 10-6M) was added to the culture medium at four times. With daily observation, the percent of beating EBs was determined up to 19 days after plating.

Spontaneously beating EBs representing more than 10% of the outgrowths was investigated at three distinct developmental stages: an early differentiation phase (shortly after initiation of contractions (5+3d), an intermediate phase (day 5+9d) and terminal differentiation phase (5+12d). The spontaneous beating frequency was measured by counting the pulsation rate of CMs incubated in DMEM at 37°C on a heating plate of a phase-contrast inverted microscope (Olympus).

**Immunocytochemical staining**

The immunostaining was performed according to the recommended manufacturer’s instructions.

The contracting EBs was fixed using 4% paraformaldehyde for immunostaining. Antibodies used in this study included: α actinin, Flk-1 (VEGF receptor), beta III tubulin for the neuronal differentiation (Bouhon et al., 2005), Brachyury (mesoderm marker), Oct-4 for the presence of the undifferentiated ES cells. Following the elimination of unbound Abs by 3x PBS washings, the EBs were Hoechst stained for 10 min at RT, 3x PBS washed and then we washed with double distilled water. Cover slips with immunostained EBs were recovered and
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DIFFERENTIATION OF EMBRYONIC STEM CELLS: LESSONS FROM EMBRYONIC DEVELOPMENT

DIFERENTIEREA CELULELOR STEM EMBRIONARE: MODEL PENTRU DEZVOLTAREA EMBRIONARA

PALL EMOKE *, LICHTNER ZSUZSANNA **, BONTOVICS BABETT **,
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Embryonic stem (ES) cells, the undifferentiated cells of early embryos are established as permanent lines and are characterised by their self-renewal capacity and the ability to retain their developmental capacity in vivo and in vitro. The pluripotent properties of ES cells are the basis of gene targeting technologies used to create mutant mouse strains with inactivated genes by homologous recombination. There are several methods to induce the formation of EBs. One of them is the formation by aggregating ES cells in hanging drops, using gravity as an aggregation force. This method presents the advantage of obtaining well-calibrated EBs almost identical in size. We used at our experiment the mouse ES cell line KA1/11/C3/C8 with a normal karyotype, at 14th passages. Immunohistochemical examination was aimed to identify tissue-restricted proteins for the two differentiated lineages: titin as a cell-specific antigen for cardiac and skeletal muscle, betaIII-tubulin for the neuronal differentiation, cytokeratin Endo-A (TROMA) for the presence of mesenchymal progenitor cells, Oct-4 for the presence of the undifferentiated ES cells. The beating cardiac muscle clumps showed more synchronous rhythm than those seen in EBs obtained from suspension culture method, where the beating cardiac muscle clumps appeared later, had a lower frequency and were uneven. The synaptic networks of neuronal cells were best developed in EBs from suspension, compared to those observed in EBs from hanging-drop method.

Key words: embryonic stem cells, in vitro differentiation, cardiac, neuronal

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**Materials and Methods**

**In vitro cultivation of mouse ES cell lines**

We used at our experiment the mouse ES cell line KA1/11/C3/C8 with a normal karyotype, at 14th passages (Fig.1). The ES cell line KA1/11/C3/C8 is a sub-clone of R1 ES cell line. R1 ES cell line was established from (129/Sv x 129/Sv-CP)F1 3.5-day blastocyst (Nagy et al., 1993). KA1/11/C3/C8 cells were kept on primary embryonic mouse fibroblast feeder layer, in Dulbecco’s modified Eagle’s medium (KO-DMEM medium)(GIBCO) supplemented with glutamax (Gibco, 100x), 50 µg/ml streptomycin (SIGMA), 50U/ml penicillin (SIGMA), 50mM β-mercaptoethanol (ME)(SIGMA), 0.1mM non-essential amino acids...
(GIBCO), 1000 units/ml of leukaemia inhibitory factor (ESGRO) and 20% fetal calf serum (FCS) (HyClone).

**Fig. 1.** ES cell colonies growing on fibroblast layer

**In vitro differentiation of ES cells into cardiac and neuronal lineages**

Two days before the differentiation, ES cells were passaged into gelatin (0.1%)(SIGMA) coated Petri dishes (Greiner) in the ES culture medium.

On the appointed day 0 of differentiation, we passage the cells into 10 cm bacteriological dishes, containing $5 \times 10^6$ ES cells in 10 ml differentiation medium (This is the Day0 of differentiation). As differentiation medium the IMDM (Gibco) medium supplemented with 0.6m/m% penicillin, 1m/m% streptomycin and 20v/v% FCS was employed. MTG (monothyoglycerol) 3 µl/ml was always freshly added to the differentiation medium. For hanging drop production $2 \times 10^7$ cells/ml containing cell-suspension was prepared in IMDM differentiation medium (**Fig.2**).

From this suspension, three Petri dish covers with 70 hanging-drops (a drop of 20 µl, contained 400 mouse ES cells) and one suspension Petri dish (3 ml cell suspension) were set in culture for two days (Day 2). Cell aggregates were obtained after 5–6 hrs, while EBs were morphologically completed after 2 days, by each procedure (**Fig.3**). The second step was to plate the EBs on a gelatin-coated surface for 17 days (Day2+7). Best EBs from the hanging-drops and from the cell suspension were separately harvested and pooled EBs from each variant were further grown on 24-well tissue culture dishes. Prior cultivation of EBs, a rounded shaped histologic cover slip was introduced within each well in order to later perform immuno-staining. All culture dishes so prepared, were gelatin-coated before addition of EBs.

For cardiomyocytes differentiation, 48 EBs were further plated in two 24-well tissue culture plates within IMDM medium/MTG. Every second day the medium was changed for 17 days. For neuronal differentiation, 48 EBs were further plated in two 24-well tissue culture plates within IMDM medium/MTG and $10^{-6}$M retinoic acid (RA). Every second day the medium was changed for 17 days, but RA was added at the first four days.
(GIBCO), 1000 units/ml of leukaemia inhibitory factor (ESGRO) and 20% fetal calf serum (FCS) (HyClone).

**Fig. 1.:** ES cell colonies growing on fibroblast layer

*In vitro differentiation of ES cells into cardiac and neuronal lineages*

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Immunocytochemical detection of specific cellular antigens proved the specificity of EBs commitment towards cardiomyocytes and neuronal lineages.

**Fig. 5.:**
Titin expression was found in beating clumps of attached EBs on gelatin-coated surface.

**Fig. 6.:**
BetaII-tubulin expression in attached EB on gelatin coated surface.

**Fig. 7.:** Cytokeratin expression in attached EB on gelatin coated surface.

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**Bibliography**


Expression of bone morphogenetic proteins (BMPs) receptors in patients with B-cell chronic lymphocytic leukemia (B-CLL)

J. DZIETCZENIA*, T. WRÓBEL*, B. JAŻWIEC*, G. MAZUR*, A. BUTRYM*, R. PORĘBA†, K. KULICZKOWSKI*

BACKGROUND

Bone morphogenetic proteins (BMPs) are the members of transforming growth factor β (TGF β) superfamily. They are multifunctional cytokines that regulate proliferation, differentiation, apoptosis, and chemotaxis in a variety of cell types including hematopoietic cells (Granjeiro et al., 2005). The function of BMPs act because of binding to two types of serine/threonine kinase receptors: BMP type I receptors (IA and IB) and BMP type II receptor. Deregulation of BMPs signaling pathways has been reported in some of human cancers, but the role of BMPs in hematopoietic malignancies remains unknown. The aim of our study was to examine the percentage of expression of BMPs receptors on lymphocytes of patients with B-cell chronic lymphocytic leukemia (B-CLL). A total of 46 patients with B-CLL (27 men and 19 women) and 10 healthy persons were evaluated. Freshly isolated mononuclear cells were incubated with antibodies against BMPs receptors: BMPRIA, BMPRIB, and BMPRII and examined in 2-color flow cytometry. On cells of patients with B-CLL, the percentage of expression of BMP RIA and BMP RIB was significantly higher than in normal cells of the control group. The percentage of the expression of BMP RIA and BMP RIB was higher in patients with advanced stage of disease.

SUMMARY

Bone morphogenetic proteins (BMPs) are multifunctional cytokines which belong to transforming growth factor β (TGF β) superfamily. They regulate proliferation, differentiation, and apoptosis in a variety of cells including hematopoietic cells. BMPs act because of binding to two types of serine/threonine kinase receptors: BMP type I receptors (IA and IB) and BMP type II receptor. Deregulation of BMPs signaling pathways has been reported in some of human cancers, but the role of BMPs in hematopoietic malignancies remains unknown. The aim of our study was to examine the percentage of expression of BMPs receptors on lymphocytes of patients with B-cell chronic lymphocytic leukemia (B-CLL). A total of 46 patients with B-CLL (27 men and 19 women) and 10 healthy persons were evaluated. Freshly isolated mononuclear cells were incubated with antibodies against BMPs receptors: BMPRIA, BMPRIB, and BMPRII and examined in 2-color flow cytometry. On cells of patients with B-CLL, the percentage of expression of BMP RIA, BMP RIB, and BMP RII was significantly higher than in normal cells of the control group. The percentage of the expression of BMP RIA and BMP RIB was higher in patients with advanced stage of disease.

Keywords

Bone morphogenetic proteins, transforming growth factor β, B-cell chronic lymphocytic leukemia
Expression of bone morphogenetic proteins (BMPs) receptors in patients with B-cell chronic lymphocytic leukemia (B-CLL)

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Murine mesenchymal stem cells isolated by low density primary culture system

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Murine mesenchymal stem cells (mMSC) and the difficult task of isolation and purification of them have been the subject of rather extensive investigation. The present study sought to isolate these cells from two different mouse strains, one outbred and the other inbred, primarily through a relatively simple but novel approach, the most important feature of which was the low density primary culture of bone marrow cells. For this purpose, mononuclear cells from either NMRI or BALB/c bone marrow were plated at about 500 cells per well of 24-well plates and incubated for 7 days. At this point, the fibroblastic clones that had emerged were pooled together and expanded through several subcultures. To investigate the mesenchymal nature, we differentiated the cells into the osteoblastic, chondrocytic and adipocytic lineages in different subcultures up to passage 10. According to the results, 1 week after culture initiation, several clones each comprising several fibroblastic cells appeared in each plate. The cells from different passages were capable of differentiating into corresponding skeletal tissues. In the present investigation, the best culture condition for maximum proliferation and also the expression of certain surface marker on isolated cells were examined. In this term the two murine strains showed some differences.

Key words: differentiation, isolation, murine mesenchymal stem cell, proliferation, surface marker.

Introduction

In addition to hematopoietic stem cells, bone marrow contains other types of stem cells, referred to by different terminology as colony forming unit fibroblasts (CFU-F), marrow stromal fibroblasts (MSF), mesenchymal stem cells (MSC) and mesenchymal progenitor cells (MPC) (Friedenstein et al. 1978; Piersma et al. 1985; Prochop 1997; Conget & Minguell 1999).

Mesenchymal stem cells, possessing two important properties of long-term self renewal and multilineage differentiation potential, have been considered as an appropriate source for cell and gene therapy-based treatment of diseases (Baksh et al. 2004). Indeed, their efficacy has been indicated in curing osteogenesis imperfecta, regenerating bone and cardiac muscle and resurfacing articular cartilage as well as restoring hematopoiesis in patients receiving chemotherapy (Koe et al. 2000; Petite et al. 2000; Quarto et al. 2001; Horwitz et al. 2002; Barry 2003; Grinnemo et al. 2004). The potential of MSC in differentiating into cells other than those of skeletal lineages, such as neurons and keratinocytes as well as liver, intestine and kidney epithelial cells, has also been demonstrated by several experiments (Sugaya 2003; Chapel et al. 2003). Such property is referred to as MSC plasticity or transdifferentiation.

In spite of the potential importance of MSC in future cell and gene therapy, not only are such biological aspects of MSC as their exact identity, developmental origin and in vivo function yet to be investigated (Bianco et al. 2001) but also their safety for transplantation purposes still remains to be clarified. Many more researches must therefore be carried out, particularly in animal models, before MSC can be used routinely for therapeutic purposes.

Mesenchymal stem cells have been successfully isolated from human, cat, dog, rabbit, rat, chicken, sheep, goat and pig bone marrows thanks to their plastic adherence property (Jessop et al. 1994; Wakisani et al. 1994; Kadiyala & Young 1997; Awad et al. 1999; Schwarz et al. 1999; Mosca et al. 2000; Ringe et al. 2000; Devine et al. 2001; Martin et al. 2002).
Colony forming unit fibroblasts assay

One hundred of the passage-4 cells were plated in a 25 cm² plastic flask in DMEM, supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin and 10% fetal calf serum before they were incubated at 37°C and 5% CO₂ for 6 days. At the end of the cultivation period, the cells were stained by crystal violet, and the number of clones was counted under an inverted microscope.

Differentiation

The passage-4 cells from each strain, having been thawed and plated at 100 cell/cm² in a 6-well plate, were incubated in DMEM supplemented with 15% FCS (proliferation medium) for 7 days until confluence was achieved. The proliferation medium was replaced with an osteogenic medium, which contained DMEM composed of 50 µg/mL ascorbic acid 2-phosphate (Sigma), 10 nM dexamethasone (Sigma) and 10 mM β-glycerol phosphate (Sigma). The cultures were then placed in an incubator at 37°C and 5% CO₂ for 21 days, with media changes three times per week. At the end of the cultivation period, the cells were fixed with 10% formalin for 10 min and stained with alizarin red (Sigma) for 15 min at room temperature, so that the mineralized matrix of the bone could be examined. The cells were also used for RNA extraction and reverse transcription–polymerase chain reaction (RT–PCR) analysis of osteocytic gene expression.

For chondrogenesis, a micromass culture technique was used. Approximately 200,000 cells (passage 4) were pelletted by centrifugation at 300 g for 4 min, followed by incubation at 37°C in 5% CO₂ in a 0.5 mL chondrogenic medium, composed of DMEM supplemented with 10 ng/mL transforming growth factor β3 (Sigma), 500 ng/mL bone morphogenetic protein-6 (Sigma), 100 nM dexamethasone (Sigma), 50 µg/mL ascorbic acid 2-phosphate (Sigma), 50 µg/mL insulin transferrin selenium (ITS) (Sigma) and 1.25 mg/mL bovine serum albumin (Sigma). The cultures were maintained for 3 weeks

RNA extraction and reverse transcription–polymerase chain reaction analysis of gene expression

Total RNA was collected from the cells having been induced to differentiate into osteoblastic, chondrocytic and adipocytic lineages as detailed above, using RNX-Plus solution (CinnaGen, Tehran, Iran). Before reverse transcription, the RNA samples were digested with DNase I (Fermentas, Burlington, Canada) to remove contaminating genomic DNA. Standard reverse transcription reactions were performed with 5 µg total RNA using oligo (dT)₁₈ as a primer and RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Subsequent PCR was as follows: 2.5 µL cDNA, 1 x PCR buffer [AMS], 200 µM dNTP, 0.5 µM of each primer pair and 1 unit/25 µL reaction Taq DNA polymerase (Fermentas). The following primers were utilized to detect osteoblastic differentiation: osteocalcin (OCN; GenBank accession number NM_007541), forward: 5′-GACCATTCTTTCTGCTACTCTG-3′; reverse: 5′-GTGATAACCATAGTGCCTGTTTAG-3′; osteopontine (OPN; accession number AF515708), forward: 5′-CAGTGTATTGTTCCTTGGCTTGTC-3′, reverse: 5′-GGTCTCCTACGACTCTGCAAG-3′; aggregan (accession number NM_011199), forward: 5′-GACAAGCTGTCAAGAAATCTTG-3′, reverse: 5′-GGAATATCCACCGGTAGATCATG-3′. The primers for chondrocytic detection were as follows: collagen II (accession number, NM_031163), forward: 5′-GGCTTAGGAGAAGAAGAAGG-3′, reverse: 5′-TGACATGGGCTCCGCTCCGCTG-3′; collagenX (accession number, NM_009925), forward: 5′-CAGACGGGGCTCCGCTCCGCTG-3′, reverse: 5′-GTGGCAAAGAAG-3′; aggrecan (accession number, NM_007424), forward: 5′-ATTGGTCAGACTTCC-3′; reverse: 5′-GACACTTGTTAAATGGTGAC-3′.

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Extrinsic Regulation of Cardiomyocyte Differentiation of Embryonic Stem Cells

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Abstract Cardiovascular disease is one of leading causes of death throughout the U.S. and the world. The damage of cardiomyocytes resulting from ischemic injury is irreversible and leads to the development of progressive heart failure, which is characterized by the loss of functional cardiomyocytes. Because cardiomyocytes are unable to regenerate in the adult heart, cell-based therapy of transplantation provides a potential alternative approach to replace damaged myocardial tissue and restore cardiac function. A major roadblock toward this goal is the lack of donor cells; therefore, it is urgent to identify the cardiovascular cells that are necessary for achieving cardiac muscle regeneration. Pluripotent embryonic stem (ES) cells have enormous potential as a source of therapeutic tissues, including cardiovascular cells; however, the regulatory elements mediating ES cell differentiation to cardiomyocytes are largely unknown. In this review, we will focus on extrinsic factors that play a role in regulating different stages of cardiomyocyte differentiation of ES cells. J. Cell. Biochem. 104: 119–128, 2008. © 2007 Wiley-Liss, Inc.

Key words: embryonic stem cells; cardiomyocyte; extrinsic factor; differentiation

Adult hearts have limited regenerative potential. Therefore, the loss of cardiomyocytes in ischemic heart disease is irreversible and results in progressive heart failure. The alternatives for treatment are limited: (i) heart transplantation is significantly hampered by inadequate numbers of donors and (ii) although several clinical trials attempted to regenerate heart muscle after a heart attack through the use of bone marrow stem cells, recent studies indicate little or no evidence of muscle regeneration from bone marrow stem cells. An alternative approach may be using embryonic stem (ES) cells as sources to generate cardiomyocyte progenitors. Transplantation of exogenous cardiomyocytes could provide functional cardiomyocytes, and therefore may be a viable therapeutic strategy to replace damaged myocardial tissue to restore cardiac function [Soloway and Harvey, 2003].

ES cells derived from the inner cell mass of the preimplantation embryo are pluripotent and capable of self-renewal. In vitro, ES cells can be cultured indefinitely, and have potential to differentiate to derivatives of all three primary germ layers. Therefore, ES cells have enormous potential as a source of therapeutic tissues, including cardiomyocytes that may be used to treat cardiovascular diseases and restore cardiac function. Because of the pluripotency, spontaneous ES cell differentiation in vitro generates multilineage cells, and only a small portion of differentiated ES cells contains contracting cardiomyocytes [Kehat et al., 2001]. A major challenge for clinical application of ES cells is to develop a differentiation protocol to generate sufficient cardiomyocytes in vitro [Sachinidis et al., 2003a]. Directing ES cells differentiation can be achieved by using extrinsic factors such as growth factors and chemicals [Schuldiner et al., 2000].

Here, we review the current status of extrinsic factors that participate in ES cells differentiation to cardiomyocytes. Among these extrinsic factors, some of them have been approved to be safe for clinical use or daily diet...
EXTRINSIC FACTORS THAT MEDIATE ES CELL DIFFERENTIATION TO CARDIOMYOCYTES

Cell functions are often triggered by extrinsic signals in environment, resulting in intrinsic changes that affect cell proliferation, differentiation, apoptosis, and migration. We will focus on two groups of extrinsic factors: growth factors and chemical compounds.

**Growth Factors**

Bone morphogenetic proteins (BMPs). BMPs are members of the transforming growth factor-beta (TGF-β) super family that play a pivotal role in most morphogenetic processes during development [Ducy and Karsenty, 2000]. BMP signalings are required in mesodermal induction and cardiac differentiation [Winnier et al., 1995; Zhang and Bradley, 1996]. Application of BMP-2 or BMP-4 to explants of cardiac region or non-cardiac regions of chick embryos induces expression of early cardiac markers, such as GATA-4 and Nkx2.5, and promotes the cardiomyocyte beating phenotype. In addition, inhibition of BMP signaling blocks expression of Nkx2.5 and cardiac differentiation [Schultheiss et al., 1997; Andree et al., 1998; Ladd et al., 1998; Yamada et al., 2000]. BMP antagonists, including noggin and chordin, truncated versions of type I (tALK3) and type II (tBMPRII) BMP receptors, and Smad6 inhibitor, inhibit cardiac differentiation [Galvin et al., 2000; Nakajima et al., 2002; Tzahor et al., 2003]. However, transient inhibition of BMP signaling prior to mesoderm development by noggin induces cardiomyocyte differentiation in mouse ES cells [Yuasa et al., 2005]. TGF-β1 is a positive factor during cardiogenesis. Behfar et al. [2002] reported that priming of mouse ES cells with TGF-β1 and BMP-2 enhanced cardiomyocyte differentiation, resulting in increased contractile regions within embryoid bodies together with increased myofibrillogenesis. Combination of activin and BMP-4 also increases cardiomyocyte differentiation from human ES cells [Laflamme et al., 2007].

The effect of BMPs on cardiomyocyte differentiation from ES cells depends on culture medium. In serum-free or low serum medium, the addition of BMP-2 and BMP-4 enhances cardiomyocyte differentiation of Cynomolgus Monkey ES cells and human ES cells, whereas BMP-4 decreases cardiomyocyte differentiation of cynomolgus monkey ES cells in FBS-containing medium [Hosseinkhani et al., 2007; Laflamme et al., 2007; Pal and Khanna, 2007]. In addition to cardiac differentiation, BMP signaling may also be essential for migration and/or fusion of the heart primordia [Walters et al., 2001]. Taken together, BMPs are essential for at least two steps in the cardiomyocyte induction process: mesodermal induction and cardiomyocyte differentiation.

Wnts. Wnts are secreted cysteine-rich glycoproteins that regulate many key developmental processes in Drosophila (Wingless, homologues to Wnts) and vertebrates, including mediation of cell–cell communication in various developmental, morphogenesis, cell fate determination, cell growth, and survival processes [Dale, 1998].

The role of Wnt signaling during cardiogenesis is dependent on the developmental stages and model system. The canonical Wnt pathway (Wnt 1, 3, 3a), which uses β-catenin as a downstream molecule, inhibits cardiomyocyte differentiation in cardiac mesoderm [Marvin et al., 2001; Schneider and Mercola, 2001; Tzahor and Lasar, 2001]. Activation of Wnt/β-catenin signaling before gastrulation promotes cardiac differentiation, but inhibits heart formation during gastrulation [Ueno et al., 2007]. Wnt/β-catenin signaling is activated at the inception of mammalian cardiac myogenesis, and is indispensable for cardiac differentiation in P19 embryonic cells [Nakamura et al., 2003]. These studies suggest that Wnt/β-catenin signaling play a biphasic role in cardiomyocyte differentiation: activation is required to commit mesenchymal cells to the cardiac lineage; downregulation of β-catenin is needed for cardiomyocyte differentiation at later stages. Activation of Wnt/β-catenin during early EB formation enhances mouse ES cell differentiation into cardiomyocytes and suppresses the differentiation into hematopoietic and vascular cell lineages [Naito et al., 2006]. It will be important to test whether the addition of wnt antagonists and agonists at different time points can direct cardiomyocyte differentiation during ES cell differentiation.

Fibroblast growth factors (FGFs). The FGFs and FGF receptors (FGFRs) have been implicated in a variety of physiological and pathological conditions, including mesodermal development, tissue growth and remodeling, inflammation, tumor growth, and vascularization [Xu et al., 1999; Powers et al., 2000]. During development, commitment to a mesodermal
Assessment of Growth Factor IGF in Cardiac Differentiation of Mouse Embryonic Stem Cells

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Abstract
Pluripotent embryonic stem cells (ES) are harvested from the inner cellular mass (ICM) of the preimplantation embryos on E3.5 and cultured thereafter in vitro. ES cells have the ability to remain undifferentiated and proliferate in vitro while maintaining the potential to differentiate into derivatives of all three embryonic germ layers including cardiomyocytes thus becoming an alternative source of functionally intact cardiomyocytes for the treatment of cardiovascular diseases. We used at our experiment the mouse ES cell line CD1EGFP (mouse embryonic stem cells line, gift from, Gocza Elen, Genetic Modification Group, Agricultural Biotechnology Center, Godollo, Hungary) with a normal karyotype, at 12th passages. Is known that insulin-like growth factor-1 (IGF-1) promotes myocyte proliferation. The purpose of this study therefore focused on assessing the effect of IGF on cardiac differentiation of mouse embryonic stem cells. Our results highlight an increase of embryoid bodies with spontaneous contractions and also a positive effect after treatment with cardioactive substances.

Key words: stem cells, differentiation, cardiomyocytes, insulin growth factors

Embryonic stem cells (ESCs), are totipotent cells derived from the inner cell mass of developing blastocysts, are established as permanent lines and characterized by their self-renewal capacity and the ability to retain their developmental capacity in vivo and in vitro (1).

ESCs cells cultivated as embryo-like aggregates, called embryoid bodies (EBs), differentiate in vitro into cellular derivatives of all three primary germ layers of endodermal, ectodermal, and mesodermal origin. Ability of stem cells to differentiate specific depends on a specific set of growth factors, signaling molecules, proteins from the extracellular matrix (ECM) (2,3,4).

Insulin-like growth factor I (IGF I) is essential for normal embryonic growth in mice and was implicated in formation of a functional heart (5,6). IGF-1 induces expression of a number of cardiac-specific transcription factors such as the zinc finger GATA proteins and Nkx-2.5, a coactivator of GATA-4 (7).

The present study we investigated the IGF-1 potential to induce cardiac differentiation of ES cells in the presence of embryoid body formation.

Materials and methods
CD1EGFP cells, kindly provided by ABC (Genetic modification Group, Hungary), were cultured with feeder cells (primary mouse embryonic fibroblast) in Knock'out DMEM (Sigma) supplemented with glutamax (Gibco, 100x), 50 µg/ml streptomycin (SIGMA), 50U/ml penicillin (Sigma), 50mM β-mercaptoethanol (ME) (Sigma), 0.1mM non-essential amino acids (Gibco), 1000 units/ml of leukemia inhibitory factor (Esgro) and 10% ES cell tested fetal calf serum (FCS) (HyClone). For induction and embryoid bodies (EBs) formation,
the ESCs were dissociated and resuspended in differentiation medium Iscove's Modified Eagle Medium (IMDM) supplemented with 0.6m/m% penicillin, 1m/m% streptomycin and 20% FCS was employed. MTG (monothyoglycerol) 3μl/ml was always freshly added to the differentiation medium. EBs was formed in hanging drops of without LIF (400 cells in 20 μL of medium). After 4 days, EBs were plated on gelatin-coated dishes and cultured for 5 days.

IGF-1 (Recombinant Human Insulin-like Growth Factor – 1), (Gibco) was added to culture medium at two final concentrations 10ng/ml and 20 ng/ml. Cultures were examined daily and the percent of beating EBs was recorded for 3 weeks after plating, in both control and IGF-1 treated groups.

Spontaneously beating EBs was investigated at three distinct developmental stages: an early differentiation phase (shortly after initiation of contractions (5+3d), an intermediate phase (day 5+9d) and terminal differentiation phase (5+12d). To assess the functionality of cells derived by differentiation the cultures were treated with chronotropic substances namely Isoprenaline, Phenyleprine and Carbachol, at three developmental stages. For evaluating the cardiac differentiation, the contracting EBs was fixed using 4% paraformaldehyde for immunostaining. Antibodies used in this study included: VEGF, GATA-6 and alfa actinin.

**Results and discussions**

In the study two concentrations of IGF-1, 10, 20 ng/mL, were administered to promote differentiation into cardiomyocytes. As a preliminary stage of differentiation the CD1EGFP cells were aggregated to obtain embryoid bodies. The EBs are able to differentiate into embryonic germ layers (endoderm, ectoderm and mesoderm). The principle of obtaining EBs is based on preventing ES attachment to the cultivation surfaces. Standard methods of prepare EBs are the hanging drops method and in static suspension culture to allow small scale formation of aggregates (8). These culture systems are capable to maintain a balance between ESCs cell aggregation and prevention of EBs agglomeration (8,9).

After treatments in both groups (treated and untreated) were identified spontaneous and rhythmic contractile activity in the early stage of differentiation (2-4 days after cultivation on gelatin coated plates). Cells with spontaneous contraction are located within a well defined portion.

After advancing the differentiation period EBs treated with 20 ng/ml IGF-1 are increasing portion size of EBs with spontaneous contraction, which towards the terminal stage of differentiation including almost all EBs. At EBs treated with 10 ng/ml IGF-1 were observed a decrease of EBs with spontaneous differentiation at terminal stages, same behavior were observed for untreated EBs. The frequency of spontaneous contractions in cardiomyocytes in the experimental groups was lower (26.25-36.25%) than the control group (50.0% - 86.0%). Compared with concentration of 20ng/ml of IGF-1 the concentration of 20 ng/ml of IGF-1 was most effective to induce differentiation (fig.1).
To evaluate the functionality and pharmacological response of ESCs-derived cardiomyocytes, the EBs (n=25) with spontaneous beating cardiomyocytes in both control and IGF-1 groups were treated with a $10^{-5}$ M concentration of isoprenaline, β1 adrenergic receptors agonist, phenylephrine, α1 adrenergic receptors agonist, carbachol and muscarinic cholinoreceptor agonist at 3 distinct developmental stages of differentiation.

Contracting clusters from the embryoid bodies in both control and IGF-1 treatment groups reacted positive or negative chronotropic, from the early stage (day 7+3) of differentiation. The presences of cardiomyocytes in both experimental groups were also confirmed by immunocytochemistry (fig.2). Expressions of cardiac specific markers were significantly increased after treatment with IGF-1 (20ng/ml).

After treatment with cardioactive substances the rate of beating was subsequently monitored. After application of isoprenaline positive chronotropic effect was revealed in early stage of differentiation. By increasing the level of developmental stage more changes were observed in beating frequency after administration of cardioactive substances. The EBs treated with 20ng/ml IGF-1 also recorded more pronounced response to isoprenaline and a decrease of contraction after treatment with carbachol. Phenylephrine enhanced the rate of beating frequency at all the developmental stages in both groups. The control and IGF-1 treated groups showed a positive staining for VEGF, GATA-6 and alfa actinin.

Cardiac differentiation is a dynamic process consisting of complex growth factors, and various signaling pathways have been implicated in the development of specialized cardiac subtypes. The differentiation of pluripotent stem cells toward cardiomyocytes is still poorly defined. Many differentiation protocols have been described to generate cardiomyocytes from pluripotent stem cells. Some of these protocols and studies demonstrate how the exposure of various growth factors to pluripotent stem cells, at an accurate timing