Original article

The role of BMP-2 in mouse embryonic stem cells differentiation

Rolul BMP-2 în diferențierea celulelor stem embrionare murine

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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. Embryonic stem cells (ESCs), isolated from embryos are established as permanent lines with self-renewal and differentiation capacity in various specialized cell lines. We used at our experiment the mouse ES cell line KA1/11/C3/C8 (mouse embryonic stem cells line, gift from, Gocza Elen, Genetic Modification Group, Agricultural Biotechnology Center, Godollo, Hungary) with a normal karyotype, at 16th passages. Because BMP-2 (Bone Morphogenetic Protein - 2) plays a crucial role in the induction of heart formation of vertebrate embryos our study was designed to evaluate the effect of BMP-2 on mouse embryonic stem cells differentiation.

Keywords: *embryonic stem cells, in vitro differentiation, cardiac, growth factors*

Rezumat

Celulele stem embrionare au capacitatea de a rămâne nediferențiate şi pot prolifera in vitro, menținând în aceeaşi timp potențialul de a diferenția în derivatele celor trei straturi embrionare germinale. Celulele stem embrionare (CES), izolate de la embrioni sunt stabilite ca linii permanente, cu auto-reînnoire şi capacitatea de diferențiere în diverse linii de celule specializate. Pentru experiment s-a utilizat linia KA1/11/C3/C8 (celule stem embrionare murine, cadou de la Gocza Elen, Grupul de Modificări Genetice, Centrul de Biotehnologii Agricole, Godollo, Ungaria) cu cariotip normal la 16 pasaje. Deoarece BMP-2 joacă un rol crucial în inducerea formării cordului la vertebrate, studiului nostru a fost conceput pentru a evalua efectul de BMP-2 în cazul diferențierii celulelor stem embrionare murine.

Cuvinte cheie: *celule stem embrionare, diferențiere in vitro, cardiace, factori de creştere*

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Introduction

Multiple stem cell research and the isolation and stabilization of stem cells lines opened several new approaches in developmental biology of mammals (1). Embryonic stem cells are pluripotent obtained from the inner cell mass of blastocysts stages embryos (2,3,4). These cells grown on feeder cells (mouse embryonic fibroblasts) maintain their pluripotent capacity and property to generate specific cells lineages, including the germ line (1,4).

Pluripotent embryonic stem cells are harvested from the inner cellular mass (ICM) of the preimplantation embryos on E3.5 (3,5 days embryos) and cultured thereafter *in vitro* (5,6,7). The stem cells bear two main properties: unlimited self-renewal in an undifferentiated state without senescence and pluripotency.

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 (8). ESCs cell lines develop from an undifferentiated stage resembling cells of the early embryo into terminally differentiated stages of the cardiogenic, myogenic, neurogenic, hematopoietic, adipogenic, or chondrogenic lineage, as well as into epithelial, endothelial, and vascular smooth muscle (VSM) cells (9).

Ability of stem cells to differentiate specific depends on a specific set of growth factors, signaling molecules, proteins from the extracellular matrix (ECM) (9-11). Bone morphogenetic proteins (BMPs) are multifunctional cytokines, part of transforming growth factor superfamily. These cytokines play a pivotal role in most morphogenetic processes during development (12). BMP signals play a central role in vertebral mesodermal induction (13,14,15) and have a notable value in cardiac induction (11, 16). BMP-2 and BMP-4 induce ventral mesoderm formation during embryogenesis (9,17-19) and is known to play a crucial role in the induction of heart formation of vertebrate embryos (20-22).

The present study was designed to evaluate the effect of BMP-2 on mouse embryonic stem cells differentiation.

Materials and methods

1. Mouse embryonic stem cell culture

We used at our experiment the mouse ESCs cell line KA1/11/C3/C8 with a normal karyotype, at 16th passages. The ESCs 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 (5). KA1/11/C3/C8 cells were kept on primary embryonic mouse fibroblast feeder layer, in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (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).

2. Differentiation into cardiomyocytes and embryoid body formation

To induce differentiation, the ESCs were dissociated from MEF (mouse embryonic fibroblast) and resuspended in differentiation medium (IMDM) (Gibco) medium 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. For hanging drop production 2x104cells/ml containing cell-suspension was prepared in IMDM (Iscove's Modified Eagle Medium) differentiation medium. After 2 days of differentiation the embryoid bodies (EBs) were transferred to bacteriological plates and after 5 days were placed onto 0,1% gelatin- coated plates.

BMP-2 (Gibco), was added to culture medium at three final concentrations of 10, 20 and 40 ng/ml. Cultures were examined daily and the percent of beating EBs was recorded (counting was performed using inverted phase microscope) for 19 days after plating, in both, control

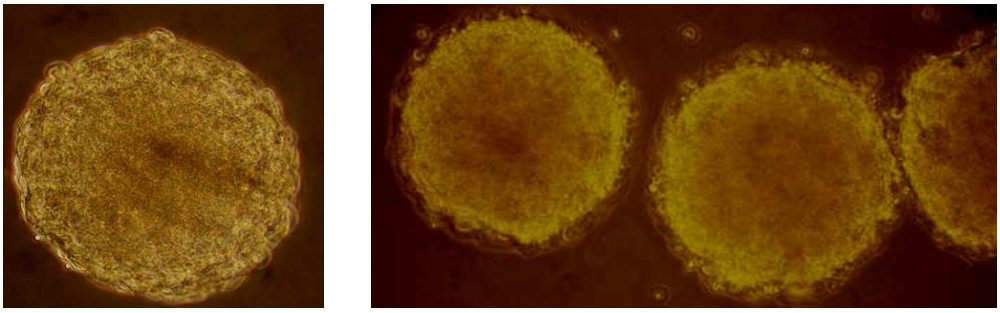

Figure 1. Morphology of aggregated embryoid bodies (EBs) in hanging drops after attachment

and BMP-2 treated groups. To assess the functionality of cells derived by differentiation the cultures were treated with chronotrop substances namely isoprenaline, phenyleprine and carbacol, at three developmental stages, early (7+3d), intermediary (day 7+7d) and in the terminal stage (7+14d). The contracting EBs was fixed using 4% paraformaldehyde for immunostaining. Antibodies used in this study included: SMA (smooth muscle actin) 1:50, titin as a cell-specific antigen for cardiac and skeletal muscle, (23) beta III tubulin for the neuronal differentiation (8), Oct-4 for the presence of the undifferentiated ES cells.

Total RNA from undifferentiated ESCs and contracting EBs of the early and late developmental stages was extracted using phenol-chloroform method.

Primer sets for cardiac α-MHC (myosin heavy chain alfa) β-MHC (myosin heavy chain beta), ANF (atrial natriuretic factor) were used in the amplification reactions. Subsequent PCR was as follows: 2.5 µl cDNA, 1xPCR buffer, 200 µl dNTP, 0,5 µl primer pair and 1 unit/25 µl reaction Taq DNA polymerase (Fermentas). Amplified DNA fragments were separated on 2% agarose gel containing 0.1µg/µl ethidium bromide and visualized under UV light.

Results and discussions

In the study were tested three different concentrations of growth factor BMP2: 10, 20 and 40 ng/ml. We studied this growth factor to evaluate the effect on differentiation of murine embryonic stem cells on cardiac line.

After treatment in both groups were identified embryoid bodies (*Figure 1*) with spontaneous contractions, in the early stage of differentiation (2-4 days after cultivation on gelatin coated plates). The frequency of spontaneous contractions in cardiomyocytes in the experimental groups was lower (25.43% - 42.35%) than the control group (69.77%), however, differences between the groups were significant only on day 1 after attachment (*Figure 2*). Compared with concentration of 20ng/ml and 40ng/ml of MBP2 the concentration of 10 ng/ml of BMP2 was most effective to induce differentiation. Myocytes-like cells were observed at the periphery of the attached EBs. The presence of cardiomyocytes

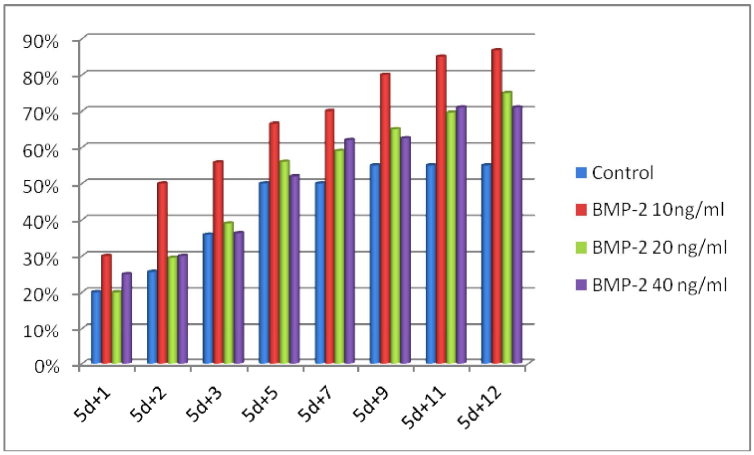

Figure 2. Percentage of embryoid bodies (EBs) with spontaneously contracting areas

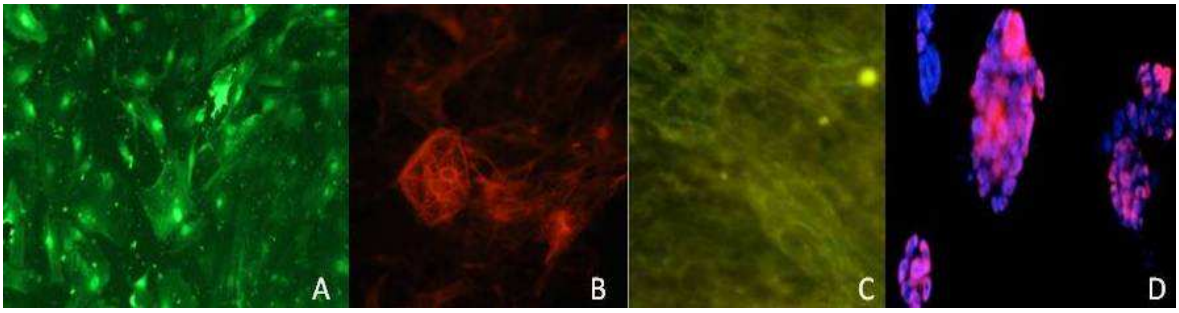

Figure 3. Immunohistochemistry highlighting A: actin-, B: titin-, βIII tubulin-, Oct-4 - positive colonies 20x (group treated with 10ng/ml BMP2)

in both experimental groups were also confirmed by immunocytochemistry (*Figure 2*). Expressions of cardiac specific markers were significantly increased after treatment with BMP2 (*Figure 3*).

The immunostaining demonstrated the expressions of proteins: actin, titin, βIII tubulin.

The genes expression study showed that the expression of α-MHC, β-MHC, ANF were increased in BMP2-treated groups more than untreated group at early and terminal stages of differentiation (*Figure 4*).

Contracting clusters from the embryoid bodies in both control and BMP-2 treatment groups reacted positive or negative chronotropic, from the early stage (day 7 + 3) of differentiation.

After treatment with cardiotropic substances the rate of beating was, subsequently, monitored. After application of isoprenaline positive chronotropic effect was revealed in early stage of differentiation. This effect was found in all tested groups.

In the intermediate stage of directed differentiation the frequency of beatings was increased compared with control group, the difference being significant ($p < 0.05$), but in the late stage of differentiation the chronotropic response was similar. Phenylephrine enhanced the rate of beating frequency at all the developmental stages in both groups. The control and BMP-2 treated groups showed a positive staining for titin, actin, ß III tubulin.

ESCs cells are pluripotent cells with self-renewal capacity. These cells can become a good model to study cardiomyocyte differentiation and the mechanisms involved in regenerative therapy (24). Previous studies have described that some cytokines can induce stem cells into specific cell types (25), including cardiomyocytes (26,27).

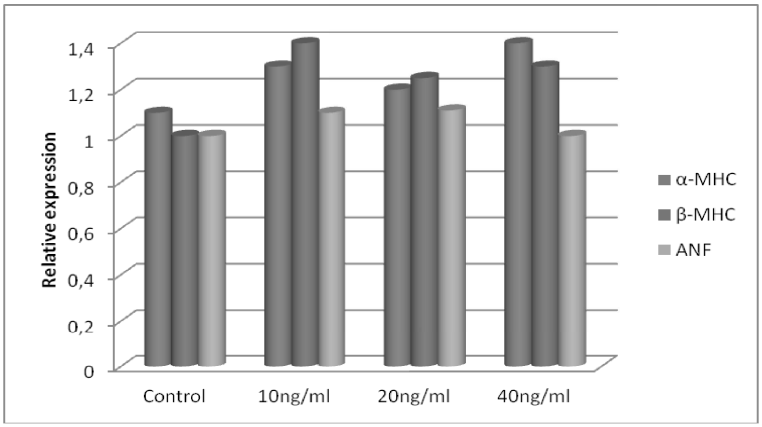

Figure 4. Relative expression of cardiac-lineage markers in the three stages of examination

Induction of ES cells into cardiomyocytes depends on a set of signaling activities (24). 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 ex-

press functional receptors typical for each cell type (28). 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 in the differentiation processes (5).

In conclusion, our results demonstrate that the treatment with BMP-2 in suspension period has an inhibitory effect on cardiomyocyte differentiation from ESCs, and also lead to a reduction in the total number of cardiomyocytes per EBs.

Our results indicate that embryonic stem cells can be efficiently differentiated into cardiomyocytes previously described by other authors (24, 29, 30). These conclusions are based on the ability of contractility, response to cardioactive drugs and specific expression of proteins, signal molecules and transcription factors.

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