IN VITRO DIFFERENTIATION OF EMBRYONIC STEM CELLS INTO CARDIAC-LIKE AND NEURONAL-LIKE CELLS

INDUÇÃO DE DIFERENCIAÇÃO IN VITRO DE CÉLULAS-TRONCO EMBRIONÁRIAS EM CÉLULAS DE TECIDO CARDÍACO E NERVOSO

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ABSTRACT

Embryonic stem cells are pluripotent cell lines with the capacity of self-renewal and a broad differentiation plasticity. They are isolated from preimplantation embryos and can be cultured *in vitro* for long time without losing their pluripotency. Embryonic stem cells can also differentiate *in vitro* with the proper combination of growth and differentiation factors, cells will differentiate into more advanced stages of embryogenesis generating different adult cell type. In the present study, we induced the *in vitro* differentiation of mouse embryonic stem cells (line R1) into cardiomyocytes and neuronal cells. These differentiations were evaluated by reverse transcription-polymerase chain reaction to verify presence of tissue-specific markers.

Keywords: *Embryonic stem cells, in vitro differentiation, embryonic stem cell culture.*

RESUMO

Células-tronco embrionárias são linhagens celulares pluripotentes capazes de se multiplicar indefinidamente e com grande capacidade de diferenciação celular. São isoladas de embriões em estágio pré-implantacional e podem ser cultivadas por longo tempo em laboratório sem perder sua pluripotencialidade. Células-tronco embrionárias podem, ainda, se diferenciar *in vitro* através da adição de fatores de crescimento e diferenciação ao meio de cultivo. As células se diferenciarão em estágios mais avançados de embriogênese, gerando tipos diferentes de células adultas. No presente estudo, induzimos a diferenciação *in vitro* de células-tronco embrionárias de camundongos (linhagem R1) em células de tecido cardíaco e nervoso. A diferenciação foi avaliada pela reação em cadeia da polimerase precedida de transcrição reversa para verificar a presença de marcadores tecido-específicos.

Unitermos: *Células-tronco embrionárias, diferenciação in vitro, cultivo de células-tronco embrionárias.*

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Several systems are currently used to study differentiation of embryonic tissues, especially embryonic stem cells (ES). ES cells were isolated for the first time from uncommitted cells of the inner cell mass of preimplantation blastocysts (1,2). This cell line shows a rapid growth in culture and proliferates indefinitely (3). Propagated under specific growth conditions, they remain in an undifferentiated state (4,5). When implanted into blastocysts, the ES cells contribute efficiently to both somatic and germ-line tissues (6). Because of the ability to generate tissues from all the three primary germ layers, ES cells are called pluripotent cells. The possibility to manipulate ES cells *in vitro*, and to introduce targeted mutations by homologous recombination, (7) promoted the possibility to generate animal models for diseases. One promising use of ES cells is the possibility to differentiate these cells *in vitro*, and later use them as a source for transplant. To achieve this aim, firstly ES cell lines have to be cultured in the presence of differentiation-inhibiting factors to maintain them in an uncommitted stage. These differentiation-inhibiting factors are provided by mitotically inactivated feeder cells, either embryonic fibroblasts (8), or by addition of leukemia inhibitory factor (LIF) to the culture medium (9). In contrast, ES cells can be stimulated to differentiate *in vitro* into different cell types by adding specific differentiation-inducing substances, *e.g.* retinoic acid (RA) or dimethyl sulfoxide (DMSO) (10,11).

To promote *in vitro* ES cell differentiation, first the ES cells must form a complex three-dimensional cell aggregate called embryoid bodies (EB). With the proper combination of growth and differentiation factors, cells within developing EB will differentiate into more advanced stages of embryogenesis, generating different adult cell types. In

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the present study, we induced the *in vitro* differentiation of mouse ES cells (line R1) into cardiomyocytes and neuronal cells. These differentiations were evaluated by reverse transcription-polymerase chain reaction (RT-PCR) to verify presence of tissue-specific markers.

MATERIAL

Cells

Mouse ES cells line R1 (12) were used as the stem cell source for the experiment. Embryonic mouse fibroblasts were extracted from fetuses of a pregnant isogenic BALB/c mice with 15 days of pregnancy.

Undifferentiated ES cell culture conditions

Embryonic mouse fibroblasts (EMFI) treated with mitomicin C were used as feeder layer. The cell culture medium used was D-MEM (GIBCO) supplemented with 15% fetal calf serum, 1% antibiotic penicillin/streptomycin, 1% LIF and 1% non-essential amino acids (NEAA) (GIBCO). ES cells were maintained in these medium and incubated in a high-humidity, 37 °C incubator containing 5% CO2 and 95% air. Every 48 hours they were splited 1:3 with trypsin ethylene diaminetetraacetic acid (EDTA) 0,5% (GIBCO) solution.

In vitro differentiation induction protocols

EB were produced applying the hanging drop method (13). EB were maintained in bacteriological dishes (non-adherent) during 7 days in Iscove's medium enriched with 15% fetal calf serum, 1% NEAA, 1% penicillin/streptomycin solution. To induce *in vitro* differentiation of ES cells into cardiac cells, we added 1% DMSO during the first 2 days of the suspension culture. To obtain neuronal cells we added 10^{-7} M RA on the third, fourth and fifth days of the suspension culture. After the seventh day of suspension culture the cells were put in culture dishes (adhesion culture) and analyzed in 3, 7, 10, 13 and 16 days.

Molecular analysis

ES cell samples obtained from days 3, 7, 10, 13 and 16 of *in vitro* cell differentiation system were collected in lysis buffer (4M guanidinium thiocyanate, 0.1 M TRIS-HCl pH 7.5, 1% β-mercaptoethanol) and contained RNA were extracted by single step extraction method (14). In parallel RNA extracted from mouse cardiac and neuronal tissue were obtained in order to be a positive control for the molecular analysis. All mRNA was reverse transcribed and amplified using oligonucleotide primers complementary and identical to the transcripts of the analyzed genes. The reverse transcription and amplification reactions were carried out with Tth-polymerase (Perkin Elmer, Norwalk, USA) following the protocol supplied by the manufacturer. β-tubulin was used as housekeeping gene and the designed primer (5'primer GGAACATAGCCGTAAACTGC- 3'primer´-TCACTGTGCCCTGAAACTTACC) was submitted to 63 ºC of annealing temperature and 40 cycles in PCR reaction. To access ES cell differentiation to cardiac cells we searched for the expression of α-major histocompatibility complex (MHC) (15).

The products of the reverse transcription reactions were denatured, followed by 35 cycles of amplification (1-minute denaturation at 95 ºC, 30-s anneling at 64 ºC and 1-minute elongation at 72 ºC). In contrast, to analyze the ES cell differentiation into neuronal cells, expression of NEURO 68 gene using specific primer (5'primer-GTTGGGAATAGGGCTCAATCT 3´primer-CCAGGAAGAGCAGACAGAGGT) was performed. The products of reverse transcription were denatured, followed by 40 cycles of amplification (1-minute denaturation at 95 ºC, 30-s anneling at 63 ºC and 1-minute elongation at 72 ºC). Additionally, the expression of synaptophysin RNA was accessed using the following oligonucleotides primers (5' primer-GCCTGTCTCCTTGAACACGAAC 3'primer-TACCGAGAGAACAACAAAGGGC). PCR technique was conduced with 40 cycles of amplification (1-minute denaturation at 95 ºC, 30-s anneling at 60 ºC and 1-minute elongation at 72 ºC). Products of RT-PCR reaction were submitted to a 2% agarose gel containing 0.35 μL of ethidium bromide and submitted to electrophoresis.

RESULTS AND DISCUSSION

Analysis of molecular biology results demonstrated the expression of β-tubulin in all mRNA analyzed samples. These data suggest that the RNA extraction method and the RT-PCR were successfully conduced.

The differentiation protocol applied to obtain cardiomyocytes from ES cells using 1% DMSO during the first 2 days of cell suspension culture demonstrated to be a simple, positive and low-cost method. Obtained cardiomyocytes presented *in vitro* morphological characteristics of cardiac tissue also expressed in the cardiac gene marker α-MHC (Figure 1) This gene encodes myosin heavy chain, a protein related to heart contraction function. It was also possible to detect a functional and physiological characteristic of cardiac tissue cells when cell cultures were analyzed by optical microscope. In addition, it was possible to observe differentiated

cardiomyocyte cluster cells promoting spontaneous contraction.

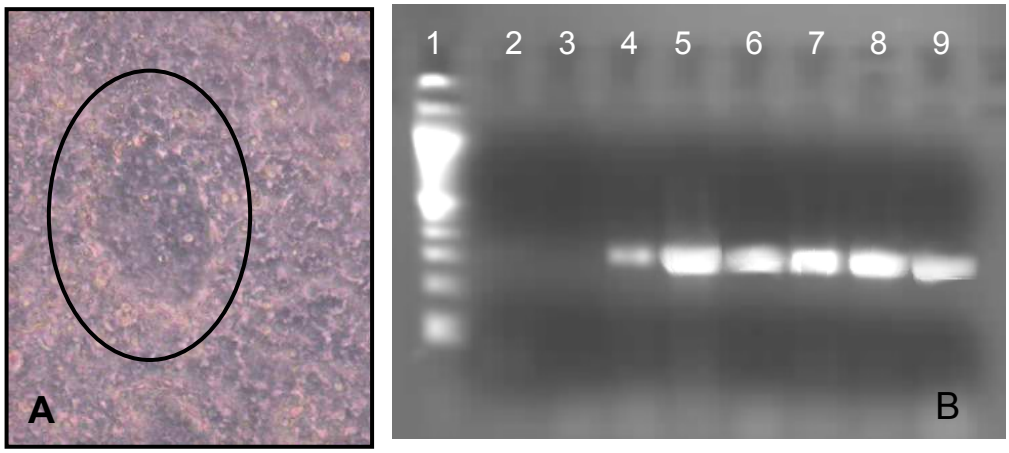

Figure 1 - Cardiac differentiation. A) Cardiac-like cell morphology after 7 days of embryonic stem differentiation protocol. In the selected area it was possible to observe spontaneous contractions resembling cardiac muscle cells. B) 2% agarose gel. Reverse transcription-polymerase chain reaction for myosin heavy chain. 1 - 100 bp DNA ladder; 2 - cDNA obtained from brain used as negative control; 3 - cDNA extracted from embryonic stem cell cultures without differentiation; 4 - cDNA obtained from differentiated embryonic stem cell (day 3); 5 - differentiated embryonic stem cells (day 7); 6 - differentiated embryonic stem cells (day 10); 7 - differentiated embryonic stem cells (day 13), 8 - differentiated embryonic stem cells (day 16); 9 - myosin gene positive control (cDNA extracted from mouse heart).

When ES cells were submitted to differentiation protocol to induce neuronal cell differentiation it was possible to observe a number of differentiated cells that showed neuronal-like morphological characteristics (Figure 2).

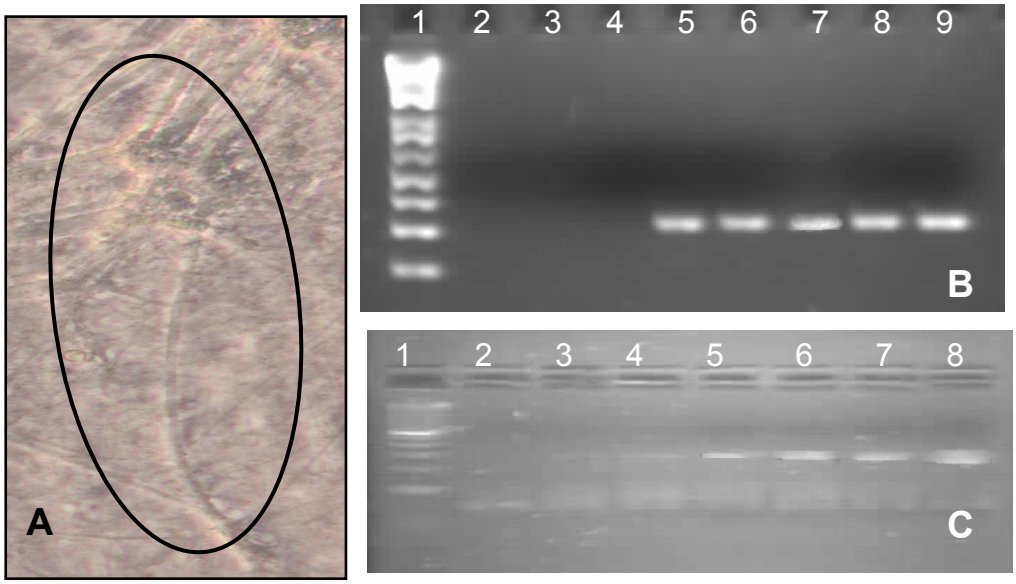

Figure 2 - Neurogenic differentiation. A) Neuronal-like cell morphology after 13 days of embryonic stem differentiation protocol. B) 1% agarose gel. Reverse transcription-polymerase chain reaction (RT-PCR) Neuro68 gene: 1 - DNA ladder 100 bp; 2 - negative control (cDNA from heart); 3 - embryonic stem cells without differentiation; 4 - differentiated embryonic stem cells (day 3); 5 - differentiated embryonic stem cells (day 7); 6 - differentiated embryonic stem cells (day 10); 7 - differentiated embryonic stem cells (day 13); 8 - differentiated ES cells (day 16); 9- positive control (cDNA obtained from mouse brain). C) 1% agarose gel. RT-PCR for synaptophysin gene: 1 - DNA ladder; 2 - embryonic stem cells without differentiation; 3 - differentiated embryonic stem cells (day 3), 4 - differentiated embryonic stem cells (day 7); 5 - differentiated embryonic stem cells (day 10); 6 - differentiated embryonic stem cells (day 13), 7 - differentiated embryonic stem cells (day 16); 8 - positive control (cDNA obtained from mouse brain).

In parallel to morphological analysis, neuronal-like cells also express the specific gene marker NEURO 68 (Figure 2). NEURO 68 gene encodes a protein from the neurofilaments, exclusively expressed by neuronal cell types. Neuronal-like obtained cells also showed the expression of the

synaptophysin gene products, which encodes a synaptic vesicle glycoprotein with four transmembrane domains. It is present in neuroendocrine cells and in virtually all neurons in the brain and spinal cord that participate in synaptic transmission (Figure 2). The synaptophysin gene expression initiated on day 7 of culture and enhanced during the culture period to the 16th day. In addition, it is possible to observe that there was no detected synaptophysin expression on day 3 of ES cells cultured with the protocol to induce neuronal differentiation. The expression of NEURO 68 and synaptophysin genes clearly demonstrated the possibility of guiding embryonic cell differentiation from pluripotent cells to neuronal-like cells, considering that it was possible to observe neuronal morphology in addition to expression of neuronal-specific proteins.

ES cells can efficiently differentiate *in vitro* into cardiomyocytes or neuronal cells. This process of *in vitro* cell differentiation can be spontaneously observed in EB, but the generated population cells is a mix of several cell types. At the moment, there is no consensus on a protocol to induce a pure cell type culture from ES cells. Trying to answer this question, many protocols have been proposed to guide the ES cell differentiation into a defined cell type. By adding specific growth factors, it is possible to increase the rate of specific cell type. We followed protocols to induce ES cell differentiation preferentially into cardiac and neuronal cell types as described by other authors (10,13,16) in order to establish the ES cell *in vitro* differentiation routine in our laboratory.

Even with the possibility of *in vitro* differentiating ES cells, there are several challenges to ES cell research and therapy (17). Some researchers have reported that long cultures of human ES cell lines do not maintain normal karyotypes (18,19). Furthermore, the formation of teratomas is associated with the undifferentiated state of ES cells. To circumvent the risk of tumor formation, protocols leading to a 100% differentiation, or efficient purification selection should be used. Methods using cell surface markers and cell sorting have been successfully tested in animals (20). Another challenge in the use of ES for therapy is the potential immunogenicity of ES cell lineages.

In conclusion, although ES cells have the potential and (as data shown here) are relatively easy and cheap to *in vitro* differentiation, there are major challenges to overcome to employ ES cells therapy.

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