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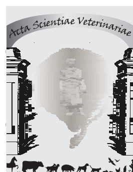

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Mouse embryonic stem cells: The establishment of the system to produce differentiated cell types *in vitro*

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ABSTRACT

During the last few years, embryonic stem (ES) cells have been a new tool in cell biology which is very promising for the scientific community to develop new cell therapies. ES cells are the only cell type that can differentiate into derivates of the three primary germ layers, not only *in vivo* but also, and most important, *in vitro*. This so-called pluripotency has resulted in the field of stem cell technology going into overdrive, and the establishment of many protocols for optimal maintenance, culture, genetic transfection and *in vitro* differentiation. The first pluripotent cells had been derived from teratocarcinomas, maligne tumors, and showed some disadvantages. Therefore later embryonic stem cells, and now adult stem cells are getting special attention from the scientists.

In this study, we established for the first time in our country, the prolonged culture of undifferentiated ES cells *in vitro* and the pointed induction of cell differentiation into specific cell types. It is the result of an international collaboration program supported by Brazil and Germany, CAPES and DAAD (PROBRAL). The well-established routine should be clearly demonstrated by the continuous culture and propagation of several mouse ES lines *in vitro* under specific culture conditions preventing differentiation. On the other hand, these ES cells were exposed to defined differentiation induction systems to obtain specialized cells as cardiogenic, neurogenic and myogenic cell types. This demonstrates the successful procedure to induce ES cell line differentiation. In this study, we established both routine systems, with and without differentiation. This results gave us competence and possibility to develop a series of different scientific approaches.

Key words: embryonic stem cell, cardiac cell, neuronal cell, myogenic cell, R1 and D3 cell lines.

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There are three types of stem cells: totipotent cells derived from the embryo, which can generate an entire organism and differentiate into all types of tissue.

Secondly pluripotent cells which can differentiate into cells of all three germ layers, but not develop to another complete individual. In the beginning pluripotent cells were isolated from teratocarcinomas, a complex type of malignant tumor containing a mixture of differentiated cell types, including derivatives of the three germ layers, and a number of undifferentiated cells, which are called embryonic carcinoma (EC) cells due to their similarity of early embryonic cells [13]. EC cells are a very good model of multilineage differentiation, but their use as a developmental model is undetermined because of their aneuploid karyotype.

Embryonic stem (ES) cells are pluripotent cell lines with the capacity of self renewal and a broad differentiation plasticity [16]. Since the first ES cell lines (sharing many features with the EC) were established from the inner cell mass (ICM) of mouse blastocysts [8] others have been derived from eighth cell embryos or dissociated from morula. ES cells are a good model for development studies, especially because ES cells do not only differentiate *in vitro* and *in vivo*, but also can be propagated as a homogeneous uncommitted cell population for an almost unlimited period of time without losing their pluripotency and their stable karyotype.

In vitro, ES cells spontaneously differentiate into many cell types, and are used to study differentiation of cardiac [11, 12, 26, 28], myogenic [17-19], haematopoietic [21, 25], epithelial [1], neuronal [2, 9, 15], vascular smooth muscle [6] and adipogenic cells [3].

Considering that this cells can be propagated with the same characteristics it can be transfected with specific gene sequences and the transgenic lines obtained should be used as a model to study the influence of the expression of that specific genes on *in vitro* and *in vivo* tissue differentiation and furthermore this transgenic ES cell lines could be injected in an embryo to analyze the influence of the expression of that specific sequence upon embryo and/or animal development.

In addition, the nucleus of an embryonic stem cell transfected with a target gene or not could be injected by micromanipulation into an enucleated zygote to produce an animal clone.

The third group are the multipotent somatic stem cells, which are derived from adult, differentiated tissues and do not present the same plasticity, capacity of long-term propagation and less ability to generate different cell types. For example haematopoietic stem cells (HSCs) isolated from the bone marrow represent only a very small amount of around 1 in 15 000 cells [24]. Beside the bone marrow cells, other adult tissues such as blood, brain, spinal cord, skeletal muscle, and epithelia of the skin and digestive system contain a similar type of multipotent somatic or adult stem cell population like the HSCs culture. But the main difference between embryo-derived pluripotent cells and soma-originated multipotent cells is that the latter are no longer able of generating germ cells and may also not differentiate into the wide range of cell types that can be derived from embryonic stem cells [5].

At the present report we decided to work with murine ES cell lines to induce *in vitro* differentiation into cardiogenic, myogenic and neurogenic cells using specific protocols established during the last years.

MATERIALS AND METHODS

Cell lines

The mouse embryonic stem cell lines D3 [4] and R1 [14] were cultured in an undifferentiated state on feeder layer cells of primary mitomycin-treated embryonic mouse fibroblasts in DMEM (Dubco's modified Eagles medium, GibcoBRL Life Technologies, GmbH) supplemented with 15% FCS, 1% non essential amino acids, 0.1% β-mercapto ethanol, 1% L-glutamine and 1% LIF as described by Wobus *et al*. 1984

***In vitro* differentiation**. To initiate *in vitro* differentiated ES cells were passaged onto gelatin-coated plastic petri dishes 24h before further treatment and cultured without LIF for 12h. Then ES cell colonies were disaggregated using trypsin solution (0.2% Gibco BRL in PBS, phosphate-buffered saline) and counted using a Neubauer's chamber. Depending on the supposed direction of differentiation between 400 and 600 ES cells/20 μl were cultured in hanging drops for two days. Within this period the cells aggregate and generate so-called "embryoid bodies" (EB). During this period we used DMEM culture medium supplemented with 15% of DCC-FCS (FCS treated with dextrancoated charcoal to remove endogenous retinoids and hormones). Afterwards EBs were cultured in suspension

using bacteriological petri dishes for additional 3 days. Subsequently EBs were plated individually in 24-well plates, in DMEM supplemented with 15% fetal calf serum (FCS; selected batches, Gibco), 1% L-glutamine (Gibco, 2 nM), 0.1% β-mercaptoethanol (Serva, Heildelberg; final concentration $5x10^{-5}$ M) and 1% non-essential amino acids (NAA; Gibco) as described [26,11].

Cell culture - For differentiation system

Cardiogenic differentiation: R1 ES cells; culture medium D-MEM (Dubco's modified Eagles medium, GibcoBRL Life Technologies, GmbH) with the supplements described above plus 1% DMSO (Gibco), 400 ES cells/20 µl microdrops, suspension culture for 7 days.

Neurogenic differentiation: D3 ES cells, Iscove's medium supplemented as described above to the D-MEM plus retinoic acid (RA) 10^{-7} M, daily supplemented during the first 3 days of 7 days suspension culture. Cell density: 800 cells/drop.

Myogenic differentiation: D3 ES cells, D-MEM supplemented as described above. Suspension culture for 5 days in total, while cell density in hanging drops was 800 cells/20 µl.

RESULTS

The ES cell lines cultured under specific, well defined differentiation-inducing conditions developed from an undifferentiated stage resembling pluripotent cells of the early embryo into terminally differentiated stages of cardiomyocytes, myocytes, and neuronal cells. These cells were analyzed by cell morphology and also physiological properties, *e. g.* the *in vitro* differentiated cardiomyocytes resembled characteristics of atrial, ventricle, spontaneously beating.

It was found that parameters like number of cells in hanging drops, media and supplementations, ES cell lines and the time of EB plating influenced the developmental potency and differentiation features of ES cells in culture.

DISCUSSION AND CONCLUSIONS

For example, RA induces differentiation of ES into specific cell types, in a time and concentration dependent manner [3, 7, 9, 10, 20, 22, 23, 27].

In this study we have successfully established for the first time in Brazil the embryonic stem cell technology including prolonged culture of various pluripotent ES cell lines as well as the directed induction of *in vitro* differentiation into a variety of specialized cell types like myogenic, cardiac and neuronal cells.

The present results gave us conditions to develop the ES cells routine in our University in order to apply this new technology to other projects where we will have conditions to analyze the influence of specific genes expression in tissue and animal development as well as we can collaborate with the groups of our University that dominate the animal reproduction technology to produce transgenic animals that should function as bioreactors to produce specific proteins.

Although the recent success in establishing human ES cells raised numerous public discussions concerning ethical, legal and political issues of this technique based on the idea to manipulate human embryos in order to introduce therapeutical cloning of human embryos used as sources of undifferentiated cells which subsequently will be used to generate autologous specific cell types *in vitro* for cell transplantation without any risk of rejection or any other side effect. Currently at the moment this topic is discussed very intensively and controversially around the world and it is necessary to define limits based on public and scientific local agreements.

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