

DIFFERENTIATION OF EMBRYONIC STEM CELLS: LESSONS FROM EMBRYONIC DEVELOPMENT

DIFERENTIAREA CELULELOR STEM EMBRIONARE: MODEL PENTRU DEZVOLTAREA EMBRIONARA

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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 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

Introduction

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 (*Wobus et al., 1984*).

ES 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, 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 in the differentiation processes (*Wobus et al., 2005*).

There are several methods to induce the formation of EBs. One of them 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. When grown as EBs, ES cells spontaneously differentiate into many cell types, including cardiomyocytes. ES cells may differentiate into endothelial progenitors, positive for early markers, such as CD31, also known as platelet-endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial growth factor receptor (VEGFR)-2. The addition of angiogenic factors in the medium, such as endothelium-specific VEGF (or VEGF-A or VEGF-165) efficiently promotes the differentiation of ECs and their organisation into vascular structures, which may contain haematopoietic cells (*Strübing et al., 1995*).

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).

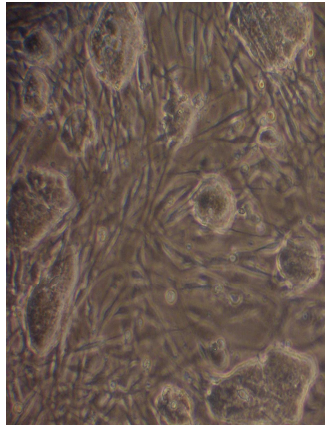


Fig. 1.: ES cell colonies growing on fibroblast layer

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 5×10^6 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 (monothoglycerol) 3 μ l/ml was always freshly added to the differentiation medium. For hanging drop production 2×10^4 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 μ l, 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.

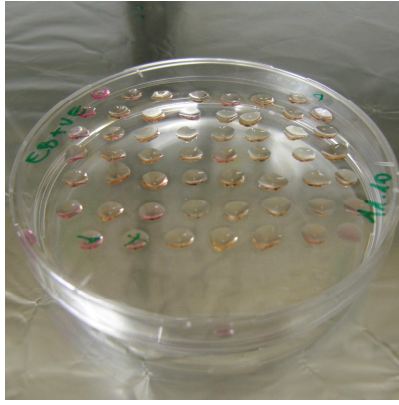


Fig. 2.: Hanging drop differentiation method.

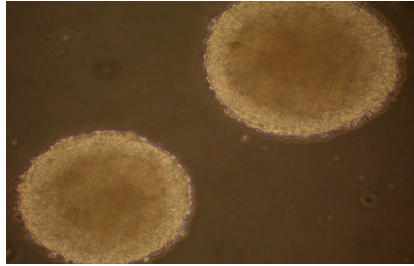


Fig. 3.: EBs at Day2+2 Hanging drop differentiation method was used.

Immunohistochemical analysis of differentiated ES cells

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, (Wobus *et al.*, 2005) betaIII tubulin for the neuronal differentiation (Bouhon *et al.*, 2005), cytokeratin Endo-A (TROMA) for the presence of mesenchymal progenitor cells, Oct-4 for the presence of the undifferentiated ES cells.

Briefly, the EBs were fixed for 10 min with 4% paraformaldehyde, washed 3x with PBS, 5 min with 0.1% Triton X-100, 45 min in the blocking solution at RT, followed by added the primary antibody. As the primary antibodies (Ab) we used mouse IgG anti betaIII-tubulin (Promega) 1:2000, mouse IgM anti Titin (Hibridoma) 1:3, goat IgG ani Oct4/3 (RD-SYS) 1:10, mouse IgG2a anti Cytokeratin Endo-A (Hibridoma) 1:3. After 24 hrs at 4°C, the EBs were washed 3x with PBS and the labelled (secondary Ab) were added for 1 hr at 37°C. The secondary Ab were anti mouse IgG-Cy3 for the betaIII-tubulin and TROMA, anti mouse IgM-FITC for titin and anti goat IgG-Cy3 for Oct4/3. The immunostaining was performed according to the recommended manufacturers' instructions. Following the elimination of unbound secondary 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 placed on Vecta-Shield Mounting Medium on chemically clean microscopic slides and examined with a fluorescence microscope.

Results and Discussions

Cardiomyocytes differentiation

From the 7th day of plating (Day2+7) we could observed contractions in a synchronous rhythm (*Table 1*)(*Fig.4*). Best contracting cells, showing titin expression (*Fig.5*), were observed within the EBs grown in IMDM medium derived from the hanging-drop variant, compared to EBs from suspension culture. The EBs from the suspension variant were smaller and the pulsing was retarded and uneven compared to the hanging-drop variant. In the RA treated plates the overall contractions of EBs were present, but at a lower frequency than in the IMDM medium, while contractions within EBs from suspension culture were only rare noticed.

Table 1.: Proportion of pulsing EBs in the four culture variants

Examination day	Pulsing EBs (%)			
	IMDM hanging-drops	IMDM Suspension culture	IMDM + RA hanging-drops	IMDM + RA Suspension culture
Day2+7	47.82	25	0	0
Day2+8	78.26	50	47.82	0
Day2+9	91.3	62.5	65.21	0
Day2+10	91.3	62.5	65.21	0
Day2+11	95.65	83.33	91.3	17.39
Day2+12	95.64	83.33	91.3	17.39
Day2+13	95.65	87.33	91.3	17.39
Day2+14	95.65	87.5	91.3	17.39
Day2+15	86.95	76.19	91.3	17.39

Neuronal differentiation 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 (Stübing et al., 1995). Committed neuronal lineage was 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.6*) and cyokeratin Endo-A (*Fig.7*). However, within EBs there were present several undifferentiated, pluripotent cells expressing the Oct-4 marker.

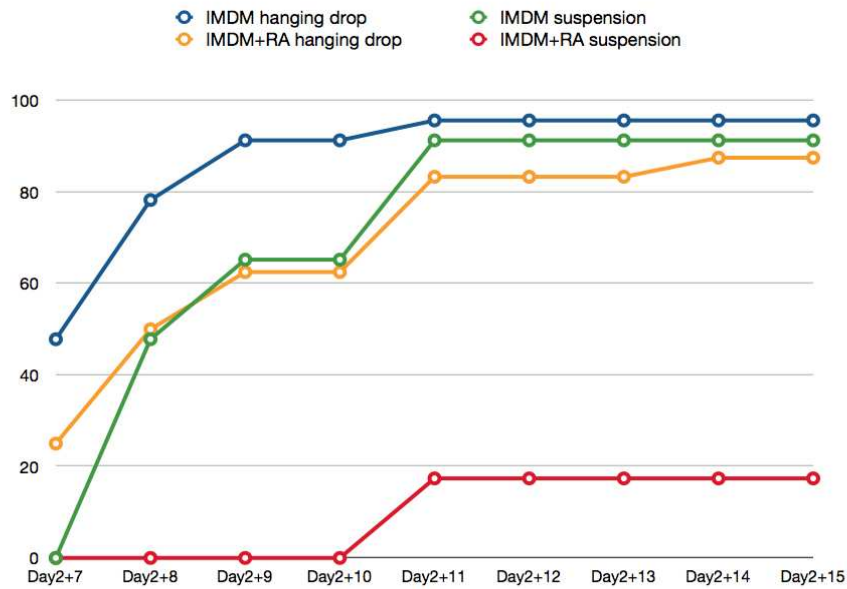


Fig. 4.: Percentage of pulsing clumps in four different culture conditions.

Conclusions

Hanging-drop method provides a practical way of generating single EBs from a defined number of cells. EBs from the suspension culture method were smaller and unequal in size, compared to the hanging-drop procedure. Cardiomyocytes began to contract spontaneously seven days after plating of EBs (Day2+7). They were localised between an epithelial and a basal mesenchymal layer. The hanging-drop method showed better results on the field of cardiac differentiation. 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.

When RA was added to the culture medium, we could observed contracting clumps in EBs obtained by the hanging-drop method later then without RA treatment (Day2+8), while in EBs obtained by suspension method contractions appeared later (Day2+11) and less frequently. Addition of RA induced committed neuronal lineage seen in EBs from the suspension variant 12 days after plating (Day2+12). The synaptic networks of neuronal cells were best developed in EBs from suspension, compared to those observed in EBs from hanging-drop method.

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

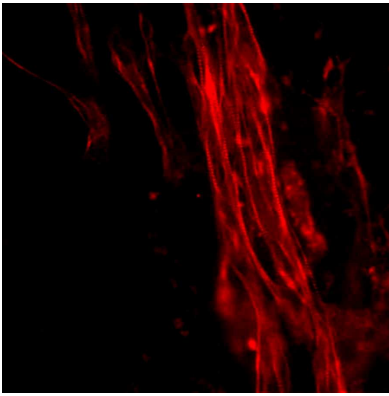


Fig. 5.:
Titin expression was found in beating clumps of attached EBs on gelatin-coated surface.

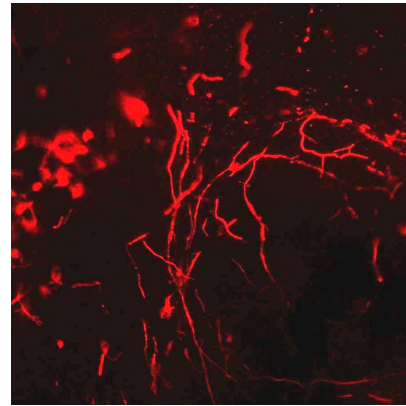


Fig. 6.:
BetaII-tubulin expression in attached EB on gelatin coated surface.

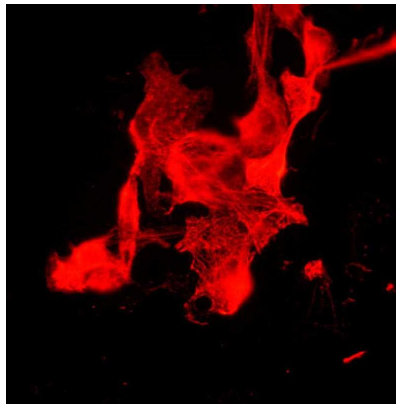


Fig. 7.: Cyokeratin expression in attached EB on gelatin coated surface.

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