Neural Differentiation of Embryonic Stem Cells is Induced by Signalling from Non-Neural Niche Cells

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Key Words

Stem cell • Differentiation • Environment • Co-culture • Humoral • Cell contact • A2B5 • Neurofilament • Nestin • Oct-4

Abstract

Background/Aims: Embryonic stem cell (ESC) transplantation offers new therapeutic strategies for neurodegenerative diseases and injury. However, the mechanisms underlying integration and differentiation of engrafted ESCs are poorly understood. This study elucidates the influence of exogenous signals on ESC differentiation using *in vitro* modelling of non-stem/ stem cell interactions. **Methods:** Murine ESCs were co-cultured with endothelial cells and astrocytes or conditioned medium obtained from endothelial or astrocyte cultures. After 7 days of co-culture isolated RNA was analysed using RT-PCR for the expression of pluripotency marker *oct-4*, neural progenitor marker *nestin*, and *neurofilament (NFL)*, an early marker of neuronal lineage commitment. The presence of the glial cell surface marker A2B5 was determined in ESCs by flow cytometry. **Results:** Neuronal differentiation was inhibited in ESCs when grown in close vicinity to cerebral endothelial or glial cells.

Under these conditions, ESC differentiation was predominantly directed towards a glial fate. However, treatment of ESCs with endothelial cell- or astrocyte-conditioned medium promoted neuronal as well as glial differentiation. **Conclusion:** Our results indicate that ESC fate is determined by endothelial and glial cells that comprise the environmental niche of these stem cells *in vivo*. The direction of differentiation processes appears to be dependent on humoral factors secreted by adjacent cell lines.

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Introduction

Pluripotent embryonic stem cells (ESCs), derived from the inner cell mass (ICM) of pre-implantation embryos, possess the ability to differentiate into all three embryonic germ layers [1, 2], and exhibit an indefinite replicative potential. These unique attributes make them one of the most promising therapeutic tools for cell replacement therapies. ESCs can be induced to differentiate *in vitro* to generate all cellular phenotypes including hematopoietic/vascular cells [3], muscle cells [4], adipocytes [5], chondrocytes [6], pancreatic islets [7], myocytes [8] and osteoblast [9] by the addition of

cytokines, hormones or other factors. Current research has primarily focused on the use of ESCs to generate neural cell lineages for the treatment of neuronal cell loss associated with neurodegenerative diseases (such as Parkinson's or Alzheimer's disease), stroke or CNS injury.

Various studies have demonstrated the successful transplantation of stem cells or precursors into different experimental models of CNS injury and disease, resulting in improvements and/or functional recovery [10-15]. However, other studies have encountered obstacles which may partly be due to an unsupportive microenvironment and the absence of appropriate cell-cell interactions [16, 17]. Therefore, the determination of the inductive or inhibitory mechanisms responsible for the survival and differentiation of transplanted ESCs remains a focal point of ongoing research.

Although it was shown that neural stem cells reside in close community with astroglial and microvascular endothelial cells in the brain and that this so-called niche plays an important role in the maintenance of NSC plasticity *in vivo* [18-20], little is known regarding the identity of environmental cues that influence stem cell differentiation in the CNS. Recent studies concerning the nature of microenvironmental signalling have demonstrated that neuronal precursor cells (NPCs) successfully differentiate into neurons when transplanted into sites associated with ongoing neurogenesis (subventricular zone and hippocampal dentate gyrus) [21-23], while the incorporation of NPCs into other hippocampal or spinal cord regions resulted in the restriction to glial phenotypes [21]. It appears likely that a combination of both extracellular signals and intrinsic factors exert a significant influence on the fate of pluripotent ESCs. Tsai et al. [24] reported that multipotent cortical stem cells, which normally gave rise to neural phenotypes under high-density culture conditions, differentiated predominately into smooth muscle cells at low density. Thus, cell-cell interaction seems to have an important effect on cell fate determination, providing the first evidence for short-range regulatory mechanisms of stem cell differentiation. Few *in vitro* co-culture models have been established to investigate the complex interplay of cell-intrinsic and extracellular signals responsible for cell fate determination. The utilization of hippocampal astrocyte feeder-layers [25] or astrocyte-conditioned medium [26-28] has proven to be supportive for neuronal cultures and exemplifies the importance of the neuron-glial relationship. An *in vitro* study by Kornyei et al. [29] demonstrated that neurogenesis in ESCs is indeed induced through interaction with glial cells. A similar study by Shen et al. [30] more intriguingly showed

that endothelial cells, which *in vivo* reside close to neural stem cell niches of the brain, are able to induce neurogenesis in ES cells. This data strongly indicates that endothelial as well as glial cells which are commonly found in close vicinity to *in vivo* neural stem cell niches play an important role in guiding ESC fate.

Although these observations provide some information regarding the nature of cues guiding stem cell fate, we are far from a complete understanding of the complex molecular regulation of cell lineage commitment, and the need remains to elucidate the coordinated molecular signalling systems that regulate stem cell fate in order to develop therapeutic approaches to treat human neurological disease.

In the present study, we firstly established a novel co-culture model that reproduces *in vivo* cell interactions to delineate the signalling cues that affect pluripotent ESC fate by assessing the roles of soluble/diffusible factors, ECM-associated molecules and the cellular environment. Co-culturing of brain-derived differentiated cells (astrocytes, endothelial cells - cell types contributing to the neural stem cell niche *in vivo*) and stem cells on opposite sides of a semi-permeable membrane allowed us to assess the interplay between cells allowing the exchange of media between the two compartments while preventing the blending of cell types. The effect of humoral factors on stem cell differentiation was determined by culturing endothelial cells and glial cells in the bottom of the well, when stem cells were grown on top of the coated filter insert. Using this model, we found that co-culturing ESCs with these brain-derived differentiated cell types results in the predominant differentiation of ESCs into A2B5-positive precursor cells. Morphological evidence of neuronal differentiation was also observed after 5 days of *in vitro* co-culture of ESCs with brain-derived endothelial cells.

Materials and Methods

Cell Culture

Rat brain endothelial cell line SV-ARBEC (NRC, Ottawa, Canada) was grown in M199 + 10% FCS + 25mg/mL peptone + 10 mL glucose (45%) (Merck KGaA, Darmstadt, Germany) + 1x BME vitamins + 1x non-essential amino acids. Immortalized rat astrocytes (glioma cell line C6; ECACC, Salisbury, UK) were cultivated in Ham's F12 + 10% FCS. The D3-murine ESC clone BAc7 transfected with green fluorescent protein (GFP) under control of a β -actin promoter [10] *in vitro* spontaneously differentiated into neural precursor cells (NPCs) when cultured according to the protocol published by Arnhold et al. [10].

In vitro pre-differentiated ESCs (NPCs) were used as differentiation control throughout the experiment.

Prior to co-culture experiments ESC were aggregated to embryoid bodies (EBs), as the arrangement of multicellular aggregates is said to aid the onset of differentiation [24, 31-33] and formation of cell-cell communication more closely resembles the *in vivo* situation [34]. Before seeding onto Transwell inserts, EBs were again dissociated in order to facilitate interaction with co-cultured cells.

Endothelial or astroglial cell-conditioned medium was prepared from DMEM medium incubated for 48 hours on confluent cell cultures. The conditioned medium was filter-sterilised and supplemented with serum-free DMEM/Ham's F12 medium (1:2). All media was supplemented with 0.5 µg/mL patricin and 0.1 mg/mL gentamycin and cells were grown at 37 °C in a humidified atmosphere containing 5% CO₂. All cell culture media and supplements were obtained from Biochrom AG, Berlin, Germany unless stated otherwise.

Isolation and culture of primary rat brain endothelial cells

Primary rat brain endothelial cells were cultured from brain microvessels isolated from 2 week old Wistar rats based on the method by Szabo et al. [35]. Endothelial cells from this preparation were grown to confluence on fibronectin/collagen IV (Sigma-Aldrich Chemie GmbH, Munich, Germany) coated Cellstar™ 6 well plates (Greiner Bio-One GmbH, Solingen-Wald, Germany) in DMEM + 20% fetal calf serum (FCS) + 100 µg/mL heparin-natrium (ratiopharm GmbH, Ulm, Germany) + 10 ng/mL bFGF (Sigma-Aldrich Chemie GmbH, Munich, Germany). Confluent cell cultures were passaged by trypsinization and used in co-culture experiments.

Co-culture

The co-culture model was established using a modification of the methods of Hurst and Fritz [36]. In brief, three different designs for co-culture experiments were chosen (Fig. 1A-C):

A) Direct co-culture (Fig. 1B). Transwell™ filter inserts (pore size 0.4 µm, diameter 1.1 cm) (Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands) were coated with fibronectin/collagen IV (Sigma-Aldrich Chemie GmbH, Munich, Germany) and endothelial cells or astrocytes were plated on the underside of the membrane in 200 µl of medium at a concentration of 5 × 10⁴ cells/mL. The cells were left to adhere overnight. The next day dissociated EBs were seeded on top of the membrane in order to allow direct interaction of adjacent ESC and endothelial / glial cells, respectively.

B) Indirect co-culture (Fig. 1C). Dissociated EBs were grown on top of the coated filter inserts, while endothelial / glial cells were seeded at the bottom of the well. In this way, factor mediated remote interaction between specified cell types was assessed.

C) Conditioned medium culture (Fig. 1A). Dissociated EBs were grown on top of the coated filter inserts in endothelial cell / astrocyte-conditioned medium. Control cultures were carried out with only dissociated embryoid bodies attached to the coated filter insert in the absence of any additional cell

Fig. 1. Co-culture settings for endothelial / glial cell - stem cell interactions. Dissociated EBs (BAC7 ESCs) were cultivated in endothelial / glial cell conditioned medium (A), opposite (B) or in close proximity (C) to endothelial cells or astrocytes, respectively. Cellular compartments were separated by a semi-permeable membrane.

type or conditioned medium. All co-cultures were grown in DMEM supplemented with 20% FCS, 50 000 I.E. heparin-natrium (ratiopharm GmbH, Ulm, Germany), 10 ng/mL bFGF (Sigma-Aldrich Chemie GmbH, Munich, Germany) for 2 days. The medium was then replaced by serum-free DMEM/Ham's F12. Thereafter, culture medium above and below the insert was carefully replaced every 48 hrs. The co-cultures were kept in a humidifier at 37 °C and 5% CO₂ atmosphere.

RNA isolation and Reverse Transcriptase Polymerase Chain Reaction Analysis

After 7 days of growth in culture, ESCs were harvested by EDTA/trypsin treatment and collected as cell pellet prior to lysis to avoid any endothelial or astroglial cell contamination. Total RNA was prepared from trypsinized ESCs grown in co-culture using High Pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). Thereafter, RNA obtained from co-cultured ESCs was examined for the expression of the pluripotency marker *oct-4*, the neural progenitor marker *nestin* and the early neuronal marker *neurofilament* (68 kDA) using semi-quantitative RT-PCR. Transcription factor *oct-4* is allocated a pivotal role in maintenance of pluripotency and early lineage commitment processes of embryonic stem cells. Thus, the assessment of *oct-4* expression is essential for the determination of stem cell phenotype. Stem cell fate is governed by precise *oct-4* transcription levels, with up- and down-regulation of *oct-4* inducing divergent developmental programmes [37, 38]. While significant down-regulation of *oct-4* expression is indicative of advanced lineage commitment, initial up-regulation of *oct-4* expression indicates the onset of neuronal differentiation [38]. *Nestin* mRNA expression, on the other hand, is closely correlated with a mitotically active bi-potential NPC state in the developing CNS *in vivo* from which glial and neuronal cell emanate. Thus, *nestin* gene expression marks a developmentally decisive stage during neuronal development. During the transition from a proliferating to a post-mitotic cell state, which is associated with changes in intermediate filament gene expression, a rapid decrease in *nestin* mRNA expression is observed [39, 40]. Loss of *nestin* expression is therefore associated with early events in either neuronal or glial lineage commitment [41]. In contrast, neurofilament proteins (light, middle and heavy chain) are expressed in a temporal pattern during embryonic development and define different degrees of neuronal lineage commitment [42]. Terminal neural differentiation has been demonstrated to involve a down-regulation of *nestin* and induction of *neurofilament* expression [40].

Reverse transcription and polymerase chain reaction amplification was performed from 20 ng template RNA using SuperScript™ One Step RT-PCR System (Invitrogen GmbH, Karlsruhe, Germany) according to manufacturer's instructions and a Biometra® Tpersonal thermocycler (Biometra, Göttingen, Germany). The sequences of the gene specific primers were as follows: the sense primer 5'-AGA GGG AAC CTC CTC TGA GC-3' and the antisense primer 5'-CTG GGA AAG GTG TCC CTG TA-3' for *oct-4*; the sense primer 5'-CTC GGG AGA GTC GCT TAG AG-3' and the antisense primer 5'-ATT AGG CAA GGG GGA AGA GA-3' for *nestin*; the sense primer 5'-TGAGCT GAG AAG CAC GAA GA-3' and the antisense primer 5'-TTG GTT GGT GAT GAG GTT GA-3' for *neurofilament* and the sense primer 5'-AGA ACA TCA TCC CTG CAT CC-3' and the antisense primer 5'-CCT GCT TCA CCA CCT TCT TG-3' for *glyceraldehyde-3-phosphate dehydrogenase (gapdh)*. PCR was performed with *oct-4* specific primers through 33 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds; with *nestin* specific primers through 42 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds; with *neurofilament* specific primers through 40 cycles of 94°C

for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds and with *gapdh* specific primers through 25 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 30 seconds. PCR products were separated by electrophoresis in 2% agarose (Gibco BRL, Karlsruhe, Germany) gel and visualised with imaging equipment in the presence of ethidium bromide (Sigma-Aldrich Chemie GmbH, Munich, Germany).

FACS analysis of A2B5 Expression

The expression of A2B5 protein on cell surfaces of human neural progenitor cells was evaluated to determine oligodendroglial lineage commitment. Resulting precursors cells were still able to differentiate into both oligodendrocytes and astrocytes, but not neurons [43]. In order to selectively detect oligodendroglial precursor cells in co-cultured ESCs, A2B5 protein expression was determined by flow cytometry. Co-cultured ESCs were detached from Transwell™ inserts by accutase II treatment, washed, sifted and resuspended in PBS (Mg²⁺ and Ca²⁺) (Gibco BRL, Karlsruhe, Germany) containing 5% FCS. Aliquots of 100 µL were stained/incubated with the monoclonal antibody anti-A2B5 (Chemicon Intl. Ltd., Hofheim/TS, Germany) at a dilution of 1:50 for 45 min at 37 °C in the dark. The cells were washed, resuspended in PBS and incubated with the secondary PerCP (Activated Peridinin-chlorophyll-protein Complex)-conjugated rat anti-mouse IgM (BD Biosciences, Heidelberg, Germany) at a dilution of 1:100 for 45 min at RT in the dark. The cells were washed, resuspended in 1 mL PBS and analysed in a FACScan (BD Biosciences, Heidelberg, Germany) equipped with an air-cooled 488 nm argon-ion-laser (15mW). The emitted fluorescence of PerCP was measured in log scale at 670 nm and analyses were performed using CellQuest software (BD Biosciences, Heidelberg, Germany).

Statistical Analysis

Two-tailed student's t-test was used to evaluate differences between experimental samples and their respective controls. All values are expressed as mean ± SEM and are derived from at least 3 independent experiments. Unless stated otherwise p-values < 0.05 were considered statistically significant. Single values obtained from PCR analyses were normalized to respective expression of the house-keeping gene *gapdh* [44] and subsequently, averaged. Data generated by flow cytometry was initially averaged and thereafter normalized to negative control/ background. Data is presented as relative gene / protein expression compared to gene / protein expression measured in untreated ESCs.

Results

The onset of differentiation of ESCs is delayed in co-cultures with brain-derived cells

In vitro pre-differentiation of ESCs (NPCs), resulted in significant reduction of *oct-4* mRNA expression (7% ± 4; p<0.01) as compared to a baseline expression in untreated ESCs (defined as a 100% expression), indicat-

Fig. 2. Onset of differentiation events is restricted in co-cultured ESCs. Whereas NPCs show a marked decrease of *oct-4* expression, *oct-4* expression levels in ESCs after 7 days of co-culture remain close to untreated ESCs. Expression of mRNA for *oct-4* was measured using semi-quantitative PCR. Data are means ($\pm$ SEM) and are presented as percentage of gene expression compared to untreated BAc7 ESCs. *Significant difference from untreated ESCs (** = $p < 0.01$). +Significant difference from collagen control (+ = $p < 0.05$). (black: collagen control; hatched: NPC = neural precursor cells; dark grey: C6 = astroglia; checkered: SV-ARBE = SV40-transfected artificial rat brain endothelial cells; white: pRBEC = primary rat brain endothelial cells).

ing the loss of pluripotency in these cells (Fig. 2). When cocultured with astroglia or endothelial cells, *oct-4* expression levels in ESCs were not significantly different from untreated ESCs (Fig. 2).

Neuronal lineage differentiation is inhibited in co-cultured embryonic stem cells

In ESCs grown on collagen-coated filter inserts as monoculture, *nestin* expression was observed to be significantly decreased ($35\% \pm 13$, $p < 0.05$) as compared to untreated cells (Fig. 3), indicating an effect of collagen on the regulation of *nestin* expression. The marked de-

Fig. 3. Expression of neural marker *nestin* is restrained to standard levels in ESCs grown in co-culture with brain-derived cells. NPCs display a significant increase in *nestin* expression while *nestin* levels of control cells grown on collagen alone decrease significantly. In contrast, *nestin* expression is significantly restrained in co-cultured ESCs compared to collagen control. Expression of mRNA for *nestin* was measured using semi-quantitative PCR. Data are means ($\pm$ SEM) and are presented as percentage of gene expression compared to untreated BAc7 ESCs. *Significant difference from untreated ESCs (* = $p < 0.05$). +Significant difference from collagen control (+ = $p < 0.05$; ++ = $p < 0.01$). (black: collagen control; hatched: NPC = neural precursor cells; dark grey: C6 = astroglia; checkered: SV-ARBE = SV40-transfected artificial rat brain endothelial cells; white: pRBEC = primary rat brain endothelial cells).

crease in *nestin* in ESCs was dramatically attenuated when ESCs were co-cultured with primary murine endothelial cells (direct co-culture: $87\% \pm 13$, indirect co-culture: $106\% \pm 25$, $p < 0.05$), astrocytes (indirect co-culture: $99\% \pm 20$, $p < 0.05$) or when ESCs were cultured in astrocyte-conditioned medium ($119\% \pm 23$, $p < 0.01$) as compared to ESCs cultured on collagen (Fig. 3). These observations suggest that culture of ESCs on collagen-coated filter inserts suppresses ESCs neural lineage commitment while the co-culture of ESCs with cerebral endothelial cells or astrocytes rescues ESCs from the inhibitory effect of collagen.

Fig. 4. Neuronal lineage development in ESCs is promoted by factors present in endothelial or astroglial cell-conditioned medium. Similar to NPCs expression of early neuronal marker *NFL* is significantly augmented in ESCs that were grown in conditioned medium prepared from either endothelial or astroglial cell cultures. On the other hand, *NFL* levels of ESCs grown opposite or in close vicinity to endothelial or glial cells remained at standard levels. Expression of mRNA for *neurofilament* was measured using semi-quantitative PCR. Data are means ($\pm$ SEM) and are presented as percentage of gene expression compared to untreated BAC7 ESCs. *Significant difference from untreated ESCs (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). (black: collagen control; hatched: NPC = neural precursor cells; dark grey: C6 = astrogloma; checked: SV-ARBEC = SV40-transfected artificial rat brain endothelial cells; white: pRBEC = primary rat brain endothelial cells).

On the other hand, an upregulation of early neuronal marker *NFL* was observed following growth of ESCs on collagen-coated filter inserts, whereas *NFL* expression was repressed in co-cultured ESCs. Conversely, significantly elevated expression of *NFL* mRNA was observed following culturing of ESCs in conditioned medium derived from either endothelial cell (SV-ARBEC:

Fig. 5. Oligodendroglial differentiation is induced in ESCs by co-culture with brain-derived endothelial or glial cells. In all co-cultures a significant increase of cells carrying cell surface marker A2B5 could be detected. Although the percentage of A2B5-expressing NPC and collagen control cells was also significantly increased, the number of co-cultured ESCs positive for A2B5 surpassed these levels easily. Expression of A2B5 on cell surfaces was measured by flow cytometry. Data are means ($\pm$ SEM) and are presented as percentage of A2B5-positive cells compared to untreated BAC7 ESCs. *Significant difference from untreated ESCs (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). *Significant difference from collagen control (* = $p < 0.05$). (black: collagen control; hatched: NPC = neural precursor cells; dark grey: C6 = astrogloma; checked: SV-ARBEC = SV40-transfected artificial rat brain endothelial cells; white: pRBEC = primary rat brain endothelial cells).

283% $\pm$ 50; pRBEC: 356% $\pm$ 82, $p < 0.05$) or astroglial cell cultures (218% $\pm$ 16, $p < 0.01$) as compared to untreated ESCs. Interestingly, in the case of primary endothelial cell co-cultures, mRNA expression even surpassed levels measured in NPCs (Fig. 4) which were also significantly upregulated (341% $\pm$ 39, $p < 0.001$) when compared to untreated ESCs (Fig. 4). Taken together, these results

Fig. 6. Spontaneous differentiation of ESCs to NPCs following a standard differentiation protocol (Arnhold et al. [10]. Undifferentiated Bac7 ESCs are grown on murine feeder layer (A). Embryoid bodies (EB) in suspension culture (B). In standardized differentiation culture following EB formation, immature neural cells start to separate from cellular aggregates (C). Thereafter, separated cells flatten and develop small axonal processes (D).

suggest that collagen induces neuronal lineage commitment in ESCs (downregulation of *nestin* and simultaneous upregulation of *NFL*) while the close proximity of endothelial cells or astrocytes represses differentiation events, maintaining ESCs at neural progenitor cell level (consistent *nestin* expression). Conversely, diffusible factors present in astrocyte- or endothelial cell-conditioned medium seem to have the ability to initiate neuronal lineage commitment in ESCs.

Cellular brain microenvironment induces glial differentiation

In addition expression of the oligodendroglial lineage marker A2B5 was determined by flow cytometry of co-cultured stem cells. The data presented represents the percentage of ESCs expressing A2B5 antigen following co-culture compared to untreated control ESCs. On average $5\% \pm 1$ of untreated ESCs expressed A2B5 antigen on their cell surface. In ES cells grown on collagen-coated cell culture inserts a significant increase in A2B5 protein expression was observed ($p < 0.001$) when compared to baseline values (Fig. 5). Remarkably, all ESCs co-cultured with endothelial cells or astrocytes, showed a marked and significant induction of A2B5 protein expression (4- to 8-fold increase in A2B5-expressing cells, $p < 0.01$) (Fig. 5). The most profound effects

were observed following culture of ESCs in endothelial cell (SV-ARBE)-conditioned medium ($750\% \pm 132$, $p < 0.001$) and in all ESC co-cultures with primary rat brain endothelial cells (pRBEC) (direct co-culture: $749\% \pm 303$, $p < 0.01$; indirect co-culture: $779\% \pm 318$, $p < 0.01$; conditioned medium: $825.3\% \pm 0.6$, $p < 0.001$) (Fig. 5). These results suggest that co-culture of ESCs with endothelial cells or astrocytes predominantly induces the formation of glial phenotypes from co-cultured ESCs.

Neuronal-like morphology of co-cultured embryonic stem cells

Our quantitative results obtained by RT-PCR and flow cytometry corresponded well to altered morphological appearance of co-cultured embryonic stem cells observed by phase contrast microscopy. *In vitro* differentiation of BAc7 ESCs (Fig. 6A) following an intermediate differentiation step of EB formation (Fig. 6B) led to the separation of individual cells from spherical aggregates (Fig. 6C) and subsequently to an enriched population of small, flat cells with apolar, bipolar, or short multipolar processes (Fig. 6D).

During co-culturing of ESCs single cells detached from ES cell aggregates. Within 5 days formation of axon-like structures was observed in single stem cells that were cultivated in SV-ARBE-, or pRBEC-conditioned

Fig. 7. Morphological alterations associated with differentiation processes can be observed in ESCs upon 5 day co-culture in medium conditioned by SV-ARBEC (A-C) and pRBEC (D-F) cell culture. While most cells remained small and developed bipolar or short multipolar processes, few cells appeared larger and displayed complex arborized processes.

medium (Fig. 7A-C, 7D-F respectively). Individual cells grown in isolation displayed complex arborized outgrowths (Fig. 7B, D, E) or long multipolar processes extending from cell bodies (Fig. 7A, C, F). In some cases processes even grew towards neighboring cells (Fig. 7F). Constitutive expression of GFP in BAc7 ESCs enabled cell identification in co-cultures and facilitated our ability to determine whether specific differentiation events were clearly associated with ESCs as evidenced by fluorescent microscopy. Figure 8 shows fluorescent images of rather large ESC-derived cell bodies derived from SV-ARBEC-conditioned cultures with distinct nuclei and axon-like processes extending from the cell body.

Discussion

In this study, we demonstrate for the first time that the differentiation of cultured ESCs is profoundly influenced by the type of cells that grow in close proximity, and that these inductive / restrictive effects, mediated via cell-cell contact and/or cell-cell signalling, have a dramatic impact on cell fate determination. *In vivo*, stem cells are subject to influences of the environmental niche in which they reside. Cell-cell interactions, cell-ECM contacts and the secretion of diffusible factors by neighbouring cells contribute to the character of a distinct niche

[18, 45, 46] and are known to orchestrate the maintenance of stem cell plasticity as well as influence the commitment of ESCs towards a specific fate [47-50]. Members of these distinct niches include astrocytes [25] and endothelial cells [19], which are found in close proximity to NSC pools within the adult brain. This “niche model” does not only apply to the maintenance of stem cell plasticity and fate choice during CNS development but also has important implications for cell replacement therapies in CNS injury and disease utilizing ESCs and their progeny.

In the present study, we analysed the gene expression profile of ESCs in response to different environmental “substrates”. We show that monoculture of ESCs on collagen-coated filter inserts leads to differentiation of these cells towards predominantly neural lineages as evidenced by accelerated expression of the early neuronal marker *NFL* and glial progenitor marker A2B5 with simultaneous down-regulation of the progenitor cell marker *nestin* after only 7 days. This gene expression profile indicates the stable and continuous adoption of cell fates representative of all three neural phenotypes [51, 52]. The inductive role of various ECM molecules, and, in particular, collagen IV, on differentiation of ESCs has been previously described [53-55]. In our study, we observed an inductive effect of collagen on stem cell differentiation, but this differentiation-inductive effect was not as

Fig. 8. Because of ESC's GFP labelling, observed neural morphologies could clearly be associated with differentiating ESCs via fluorescent microscopy. All pictures show ESCs cultured in SV-ARBEC-conditioned medium.

pronounced as that observed after directed pre-differentiation of ESCs towards *NFL*-expressing NPCs. The elevated transcriptional rate of *nestin*, implies that the majority of cells remain in a multipotent progenitor cell state [56, 57]. Homotypic cell contacts in areas of higher cell density within ESC populations could promote self-renewal by influencing β -catenin signalling pathways [58, 59], induce symmetric rather than asymmetric cell division [60] and/or activate Notch signalling [61], and might therefore be responsible for a delay in differentiation, promotion of growth as epithelial sheets and/or prevention of neurogenic commitment of affected ESCs [61-63].

We demonstrate also that the observed inductive effect of collagen on ESC differentiation is compensated for by the presence of cells establishing the vascular niche.

Contact and non-contact co-culture of ESCs with astrocytes (C6) and immortalised brain endothelial cells (SV-ARBEC) is known to delay cell cycle arrest by prolonged transcription of the pluripotential cell marker *oct-4* [37]. Furthermore, the neural progenitor cell marker *nestin*, was expressed at levels close to undifferentiated stem cells. Gene transcription of the early neuronal marker *NFL* was also observed in our study to be inhibited when ESCs were grown in close vicinity to environmental niche cells. However, ESCs treated with conditioned medium of all three environmental niche cell types induced significant expression of *NFL*, indicative of differentiation towards a neuronal phenotype. Similarly, a study by Shen et al 2004 [30] revealed an inhibitory role for endothelial cells on adult stem cell differentiation where neural stem cells were observed to proliferate and produce undifferentiated progeny when cultured with endothelial cells. The subsequent removal of endothelial cells induced differentiation of NSCs into neurons, astrocytes and oligodendrocytes. Likewise, it has been shown that cell-cell contact between neuroectodermal progenitor cells and astroglial cells hinders the migration of the neuroectodermal progenitor cells and instead promotes their proliferation [29]. Resulting increased intercellular contacts among neuroectodermal cells in turn facilitated neuronal lineage commitment. In our study, both astroglial and endothelial cells appear to promote proliferative rather than differentiation responses in ESCs. Increased mitotic activity of ESCs may then gradually initiate glial lineage commitment. Taken together, these data suggest that adjacent non-stem cells initially promote the continuous proliferation of ESCs in order to establish cell-cell contacts required for subsequent differentiation. Indeed, astroglial cells have been demonstrated to produce a variety of growth factors, some of which (FGF2, EGF, insulin-like growth factor binding protein 6 (IGFBP6)) have been shown to act on stem cell proliferation *in vitro* [64-67]. Removal of these factors from culture medium, results in ESC differentiation. As these factors are easily degradable [68], astrocyte-conditioned medium might no longer provide appropriate amounts of these proliferation maintaining molecules, resulting in the initiation of stem cell differentiation. On the other hand, factors such as VEGF [69, 70] and insulin-like growth factors [71] which have the ability to induce differentiation, might come into effect once factors maintaining self-renewal are degraded. It remains to be elucidated if there might even be an antagonistic action involved.

It is known that cell-cell communication is an important prerequisite for differentiation of CNS stem cells

via lateral induction / inhibition [24, 72-74], a process that is mediated by Notch signalling and involves interaction between signalling and neighboring cells. It is possible, therefore, that adjacent cells initially promote the permanent proliferation of stem cells in order to establish cell-cell contacts required for differentiation [29]. However, continuous proliferative events impact on the differentiation capacity of ESCs and although remaining multipotential, they can then give rise to higher proportions of glial phenotypes [75]. In support of this hypothesis, we detected abundant expression of the glial progenitor antigen A2B5 [43] on cell surfaces of all co-cultured ESCs. The number of ESCs expressing this cell surface marker was markedly increased compared to control ESCs that were not co-cultured with environmental niche cells. Stimulation of glial differentiation by endothelial cells or astrocytes has previously been proposed within the developing optic nerve [76, 77] and Morrow et al. [78] have demonstrated an increase in gliogenic signals produced by the developing CNS that induced embryonic cortical progenitor cells to adopt glial phenotypes at the expense of neuronal differentiation.

Taken together, our observations suggest that the proximity of non-neural cells dramatically influences the ultimate phenotype of ESCs, and that cell-cell contact or the presence of an easily degradable substance restricts differentiation and instead promote self-renewal, while the absence / dilution of this substance in conditioned medium induces neuronal lineage commitment. These find-

ings confirm previous data obtained in models of embryonic development, where increasing distance from the source of the signal (Wnt signalling during neural crest development) leads to mitotic arrest and induces differentiation [79]. We suggest that stem cell developmental fate within the CNS largely depends on distance-dependent variation in stem cell responsiveness to environmental cues and / or the differential potency of small gradients of soluble signalling molecules presented to ESCs by adjacent non-neural cells.

The differential results encountered when comparing the effects of immortalized vs. primary endothelial cells on ESC differentiation might be due to 1) for immortalized cells common, altered endothelial properties [80, 81] and 2) the difference in age of the cells. Finally, it must be noted that stem cell cultures generally represent a heterogeneous population containing stem cells in various stages of development and the data presented here may not be reflective of all ESCs.

In conclusion, the results of the present study suggest that specific intrinsic properties of ESCs, such as the establishment of intercellular communication with other non-neuronal cells in their environment, in association with exogenous soluble cues provided by the adjacent cellular milieu, dictates stem cell fate and influences self-renewal or lineage commitment. These important cues may establish a suitable microenvironment for long term maintenance of generative multipotent progenitor cells with neurogenic capacity.

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