Developmental Biology 328 (2009) 456–471

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Regulation of mouse embryonic stem cell neural differentiation by retinoic acid

Mijeong Kim [a,c], Ayman Habiba [a], Jason M. Doherty [b], Jason C. Mills [b], Robert W. Mercer [a], James E. Huettner [a,*]

[a] Department of Cell Biology and Physiology, Washington University Medical School, 660 South Euclid Avenue, St. Louis, MO 63110, USA
[b] Department of Pathology and Immunology, Washington University Medical School, 660 South Euclid Avenue, St. Louis, MO 63110, USA
[c] Program in Developmental Biology, Washington University Medical School, 660 South Euclid Avenue, St. Louis, MO 63110, USA

ARTICLE INFO

Article history:
Received for publication 9 December 2008
Revised 20 January 2009
Accepted 3 February 2009
Available online 13 February 2009

Keywords:
Telencephalon
Spinal cord
Serum-free
Transcriptional profile

ABSTRACT

Pluripotent mouse embryonic stem cells (ESCs) derived from the early blastocyst can differentiate *in vitro* into a variety of somatic cell types including lineages from all three embryonic germ layers. Protocols for ES cell neural differentiation typically involve induction by retinoic acid (RA), or by exposure to growth factors or medium conditioned by other cell types. A serum-free differentiation (SFD) medium completely lacking exogenous retinoids was devised that allows for efficient conversion of aggregated mouse ESCs into neural precursors and immature neurons. Neural cells produced in this medium express neuronal ion channels, establish polarity, and form functional excitatory and inhibitory synapses. Brief exposure to RA during the period of cell aggregation speeds neuronal maturation and suppresses cell proliferation. Differentiation without RA yields neurons and neural progenitors with apparent telencephalic identity, whereas cells differentiated with exposure to RA express markers of hindbrain and spinal cord. Transcriptional profiling indicates a substantial representation of transit amplifying neuroblasts in SFD cultures not exposed to RA.

Introduction

In vitro differentiation of embryonic stem cells (ESCs) has attracted wide interest as an experimental system for investigating developmental pathways and mechanisms. In addition, the isolation of human ESCs (Thomson et al., 1998) and human induced pluripotent stem cells (Takahashi et al., 2007; Park et al., 2008) has raised the possibility that *in vitro* differentiation may provide a novel source of cells for tissue replacement or repair (Murry and Keller, 2008). Therapeutic use of ESCs will require robust and reliable methods for producing specific neural cell types. Early work on mouse ESC *in vitro* differentiation was performed in serum-supplemented medium (Doetschman et al., 1985). These experiments found that aggregation of cells into embryoid bodies, combined with exposure to retinoic acid (RA), enhanced the efficiency of ESC conversion to a neural phenotype (Bain et al., 1995; Fraichard et al., 1995; Strübing et al., 1995). Aggregation alone in the presence of serum favours differentiation into non-neural cell types including cardiac cells (Bain et al., 1996), whereas addition of 0.5 to 1 μM RA suppresses non-neural differentiation and instead results in a high proportion of cells becoming neurons or astrocytes (Bain et al., 1995). Neurons produced in this way acquire axonal and dendritic polarity, form functional synapses, and include a mixture of excitatory cells that release glutamate as their transmitter and inhibitory cells that use either GABA or glycine (Strübing et al., 1995; Finley et al., 1996).

Because serum contains a large number of factors that might influence the differentiation process, a number of groups have investigated the *in vitro* conversion of ESCs into neurons, or neural precursors, under serum-free growth conditions (Okabe et al., 1996; Wiles and Johansson, 1999; Finley et al., 1999; Tropepe et al., 2001; Ying et al., 2003; Watanabe et al., 2005; Bouhon et al., 2005). In addition, modifications to the original differentiation procedures have been devised with the goal of enhancing production of specific neural phenotypes including dopaminergic neurons (Kawasaki et al., 2000; Lee et al., 2000), motorneurons (Wichterle et al., 2002), cerebellar neurons (Salero and Hatten, 2007) and oligodendrocytes (Brüstle et al., 1999; Liu et al., 2000). Many of these studies have used media or media supplements with proprietary composition, or they employed serum or cell-conditioned media (Kawasaki et al., 2000; Barberi et al., 2003), which makes it difficult to evaluate the specific requirements for efficient ESC growth and/or differentiation (Cai and Grabel, 2007). Moreover, it is generally recognized that a more comprehensive comparison of the differentiated cell phenotypes produced by these different *in vitro* induction procedures is desirable (Glaser and Brüstle, 2005).

A goal of our work has been to simplify the protocol required for *in vitro* neural induction while preserving cell survival and eliminating exposure to exogenous retinoids. Here we describe a serum-free, retinoid-free, growth medium supporting robust neural differentiation with insulin, transferrin and BSA as the only exogenous protein

* Corresponding author. Fax: +1 314 362 7463.
E-mail address: jhuettner@wustl.edu (J.E. Huettner).

0012-1606/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2009.02.001

constituents. Neurons derived in this medium exhibit many characteristics of those induced by retinoic acid, but transcriptional profiling revealed substantial differences in gene expression between retinoid-free versus retinoid-exposed cell populations that was confirmed by electrophysiology and immunofluorescence.

Methods

ES cell culture

Murine ESCs were propagated independent of feeder cells in 25 cm^2 tissue culture flasks that had been coated with gelatin (0.1% from bovine skin in sterile water; Sigma). The D3 and CE3 ESC lines were obtained from Dr. David Gottlieb (Adams et al., 2003); the B5 line was obtained from Dr. Andras Nagy (Hadjantonakis et al., 1998). The growth medium for dividing ESCs was Dulbecco's Modified Eagle Medium (DMEM; Life Technologies), which was supplemented with 20% calf serum, nucleosides (30 μM adenosine, cytodine, uridine, guanidine and 10 μM thymidine; Sigma), leukemia inhibitory factor (LIF, 1000 U ml^{-1} ESGRO, murine; Life Technologies) and 2-mercaptoethanol (0.1 mM; Sigma). When cells reached 80 to 90% confluence the medium was removed, the flask was rinsed with divalent-free Earl's balanced salt solution (EBS; Life Technologies), and incubated for 1 min with protease XXIII (1 mg ml^{-1}; Sigma) in divalent-free EBS. Detached cells were triturated gently, collected by centrifugation (70 ×g for 5 min), and then resuspended in 4 ml of complete growth medium. A 1:4 dilution of this cell suspension was used to seed a new flask. All cultures were kept at 37 °C in a 5% CO_2 humidified air incubator.

Neural differentiation

To initiate neural differentiation, ESCs were passaged from the flask with protease XXIII in divalent-free EBS and collected by centrifugation. Cells were resuspended in serum-free differentiation medium (Supplementary Table 1) supplemented with nucleosides (see above) and with 4 mM glutamine, and seeded at a density of 1.5 to 2×10^6 per 5 ml in 5 cm non-adhesive bacteriological Petri plates that had been coated with 0.15% agarose (Sigma, Type II-A). Under these conditions, cells remained suspended and formed aggregates that increased in size to form embryoid bodies. Every 2 d the aggregates were provided with fresh medium as follows. Medium and aggregates were transferred from the Petri dish to a 5 ml round-bottomed tube using a transfer pipette. Fresh medium was added to the empty Petri dish while the aggregates were allowed to settle in the tube for 5–10 min at room temp. Old medium was removed from the tube. The aggregates were resuspended with fresh medium from the Petri dish, transferred back into the Petri dish and returned to the incubator. For some experiments 500 nM all-*trans* RA (Sigma) was added to the medium from d4–8. After 10–12 d, aggregates were dissociated into a single cell suspension and plated onto 35 mm plastic tissue culture dishes or glass cover slips that were coated with a mixture of poly-DL-ornithine (200 μg ml^{-1}; Sigma) and mouse laminin (3 μg ml^{-1}; Gibco). Aggregates were collected in a 5 ml round bottomed tube and incubated for 5 min at room temperature in divalent-free EBS containing protease XXIII (1 mg ml^{-1}). Aggregates were rinsed 2 times with EBS containing 0.1% BSA (Sigma) and 0.1% ovomucoid (Sigma), and then once with SFD medium (Supplementary Table 1). Aggregates were triturated with a fire-polished Pasteur pipette in a total volume of 1–2 ml of SFD medium. Approximately 2–3 fold more cells were obtained from aggregates maintained in SFD medium alone for 12 d than from cultures that were exposed to RA (see Results). For plating, cells were suspended in SFD medium at a density of 3 to 6×10^5 cells ml^{-1} and dispensed onto coated dishes or cover slips. Cells differentiated in SFD medium alone were plated at lower density (~150 cells mm^{-2}) than RA-treated cells (300 cells ml^{-2}) to compensate for differences in proliferation rate (see Results). Cells were allowed to settle and attach for 1 h, then gently rinsed once with SFD medium before adding 0.5 to 1 ml of SFD medium supplemented with 0.25 mM glutamine. Cultures were fed every 2 to 3 d by partial medium replacement. For some experiments, division of non-neuronal cells was inhibited by addition of cytosine arabinoside (10 μM; Sigma) several days after plating. All inductions were performed within 10 passages of thawing from low passage number frozen stocks.

Electrophysiology

Recordings from cells with neuronal morphology were performed as described previously (Bain et al., 1995; Finley et al., 1996). Briefly, cultures were perfused at a rate of 1–2 ml/min with Tyrode's solution: 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with NaOH. Pipettes for whole cell recording were pulled from boralex glass capillaries. The internal solution used to record Na and K currents contained 140 mM KCH_3SO_3, or 140 mM K-glucuronate, 10 mM NaCl, 5 mM $MgCl_2$, 200 μM EGTA and 10 mM HEPES, adjusted to pH 7.4 with KOH. For recording agonist-evoked currents, pipettes were filled with 140 mM Cs-glucuronate, 10 mM EGTA, 1 mM ATP (Mg salt), 0.3 mM GTP (tris salt), and 10 mM HEPES, adjusted to pH 7.4 with CsOH. The open tip resistance ranged from 1 to 5 MΩ. Drug solutions were applied to the cells using an array of microcapillary tubes. The time constant for exchange of the external solution was 30–50 ms. Excitatory and inhibitory agonists were dissolved in 160 mM NaCl, 2 mM $CaCl_2$, 0.5 μM tetrodotoxin (TTX, Sigma) and 10 mM HEPES, adjusted to pH 7.4 with NaOH.

Current was recorded with an Axopatch 200A amplifier (Axon Instruments), filtered at 1 to 5 kHz (−3 db, 4 pole Bessel), and digitized at 10 to 20 kHz. Current traces were corrected for leak and capacity using scaled current evoked by an 8 to 10 mV hyperpolarizing step from the holding potential. Membrane potentials were corrected for the junction potential between the internal solution and the Tyrode's solution in which seals were formed. This potential was −10 mV for pipettes containing Cs-glucuronate. All experiments were performed at room temperature.

Immunofluorescence

ESC aggregates or monolayer cultures of differentiated cells were rinsed with Tyrode's solution and incubated for 15–20 min at room temperature in 0.1 M Na Phosphate, pH 7.4, containing 4% paraformaldehyde and for some experiments 0.1% glutaraldehyde. After three rinses with Tris-buffered saline, pH 7.4, aggregates were equilibrated with 30% sucrose, collected in an inverted pyramidal mold (Polysciences) and frozen on dry ice. Aggregates frozen in sucrose were bonded to the chuck with O.C.T. compound (Tissue-Tek), cryosectioned (6 μm) and mounted on Snowcoat X-tra slides (Surgipath). Mounted sections and fixed, rinsed monolayer cultures were incubated for 30 min at room temperature with blocking solution (BS): PBS containing 1% normal goat serum, 0.02% sodium azide, and 0.1% Triton X-100; and then incubated overnight at 4 °C (or in some cases for 2 h at room temperature) with primary antibodies diluted in BS. Samples were rinsed three times with PBS and incubated for 1 h at room temperature with fluorescent conjugated secondary antibodies diluted in BS. They were then rinsed three times with PBS and examined under epi-illumination with appropriate filters. Primary and secondary antibodies and dilutions are reported in Supplementary Table 2. Images were acquired with a Nikon Eclipse E600 microscope with 10×, 40× and 60× objectives (0.3, 0.8 and 1.0 N. A., respectively). Image analysis and quantification was performed with MetaMorph software (Universal Imaging). Most figure panels show sections that did not exceed the field of view of the camera (Photometrics CoolSNAP ES).