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All-*trans*-retinoid acid induces the differentiation of encapsulated mouse embryonic stem cells into GABAergic neurons ☆

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ABSTRACT

Embryonic stem (ES) cells are pluripotent cells that can differentiate into all three main germ layers: endoderm, mesoderm, and ectoderm. Although a number of methods have been developed to differentiate ES cells into neuronal phenotypes such as sensory and motor neurons, the efficient generation of GABAergic interneurons from ES cells still presents an ongoing challenge. Because the main output of inhibitory GABAergic interneurons is the gamma-aminobutyric-acid (GABA), a neurotransmitter whose controlled homeostasis is required for normal brain function, the efficient generation in culture of functional interneurons may have future implications on the treatment of neurological disorders such as epilepsy, autism, and schizophrenia. The goal of this work was to examine the generation of GABAergic neurons from mouse ES cells by comparing an embryoid body-based methodology versus a hydrogel-based encapsulation protocol that involves the use of all-*trans*-retinoid acid (RA). We observed that (1) there was a 2-fold increase in neuronal differentiation in encapsulated versus non-encapsulated cells and (2) there was an increase in the specificity for interneuronal differentiation in encapsulated cells, as assessed by mRNA expression and electrophysiology approaches. Furthermore, our results indicate that most of the neurons obtained from encapsulated mouse ES cells are GABA-positive (~87%). Thus, these results suggest that combining encapsulation of ES cells and RA treatment provide a more efficient and scalable differentiation strategy for the generation in culture of functional GABAergic interneurons. This technology may have implications for future cell replacement therapies and the treatment of CNS disorders.

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1. Introduction

Embryonic stem (ES) cells have the potential to differentiate into all three cell lineages (i.e., endoderm, mesoderm, and ectoderm), providing a new perspective not only for embryonic development but also for their application in cell replacement therapies (Murry and Keller, 2008; Weitzer, 2006). ES cells are derived from the inner cell mass of blastocyte-stage (day 3.5) embryos (Williams et al., 1988) and have been experimentally differentiated into various cell types including cardiac, skeletal, and neuronal cells (Choi et al., 2005). A number of studies have shown that treatment of ES cells with RA enhances their efficiency of neuronal differentiation (Bain et al., 1995; Fraichard et al., 1995; Strubing et al., 1995; see Clagett-Dame et al., 2006 and Soprano et al., 2007 for recent reviews). However, the generation in culture of GABAergic neurons is difficult and very challenging, and usually requires the employment of expensive growth factors such as basic Fibroblast Growth Factor (bFGF) and Epidermal Growth Factor (EGF) (Chatzi et al., 2009). Similarly, other strategies involve complex cell manipulations such as the generation of neural progenitors and subsequent withdrawal of mitogens (Westmoreland et al., 2001) or sequential RA treatment followed by potassium chloride depolarization (Bosch et al., 2004).

GABAergic interneurons release gamma-aminobutyric-acid (GABA), which is the main inhibitory neurotransmitter in the central nervous system (CNS). A number of neurological conditions such as Huntington's disease, epilepsy, chronic pain, anxiety and other mood disorders are associated with a remarkable dysfunctional GABAergic inhibition and neuronal hyperexcitability in the CNS (Benes and Berretta, 2001; Benes et al., 2007; Brambilla et al., 2003; Cicchetti and Parent, 1996; Kumar and Buckmaster, 2006). Because stem cell-based therapies that

Abbreviations: EB, Embryoid body; LIF, Leukemia inhibitory factor; NE, Non-encapsulated; PDL, Poly-D-lysine; RA, all-*trans*-Retinoic acid

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involve the transplantation of inhibitory interneurons are now being developed to treat a spectrum of neurological conditions, the efficient generation of GABAergic neurons in culture is germane to the success of these cell replacement strategies.

Most of the current ES to GABAergic differentiation strategies employ the generation of embryoid bodies (EBs), which are cell aggregates comprised of all three germ layers. However, these strategies possess inherent limitations that affect the efficiency of ES differentiation due to (1) a wide variability in EB size (Gerami-Naini et al., 2004), (2) aggregation among EBs in high concentration static cultures (Dang et al., 2004) and (3) limited diffusion of inducing morphogens into the EBs (Carpenedo et al., 2010), among others. A strategy currently employed to overcome this problem is the encapsulation of cells in biologically compatible hydrogels. Hydrogels composed of materials such as agarose (Bauwens et al., 2005), dextran (Doetschman et al., 1985), and gelatin (Akasha et al., 2008) have been used for culturing ES cells. However, alginic acid or alginate, a polysaccharide obtained from brown algae (Hwang et al., 2009; Maguire et al., 2006; Magyar et al., 2001), remains the encapsulation material of choice because of its intrinsic properties (Orive et al., 2003). Alginates are natural linear polysaccharides with 1,4-linked β-D-mannuronate and α-L-guluronate residues arranged as blocks of similar and alternating residues (Silva et al., 2010). Alginate is an appealing material for the construction of "biohybrid organs" and "micro-bioreactors" because its hydrated 3D network allows cells to adhere, spread, migrate and interact with other cells (Zimmermann et al., 2001). The alginate technology has also been applied to the generation of well-vascularized EBs (Gerecht-Nir et al., 2004; Magyar et al., 2001). Similarly, other researchers have demonstrated that the alginate-based encapsulation of ES cells can increase the efficiency of differentiation along various cell lineages (Bauwens et al., 2005; Hwang et al., 2009; Jing et al., 2010; Li et al., 2011; Maguire et al., 2007; Wang et al., 2009). Thus, encapsulation of cells in hydrogels represents an important method for improving current stem cell differentiation strategies.

In this work, we generated GABAergic neurons from mouse ES cells using an alginate-based encapsulation protocol. We show that up to 87% GABAergic neurons are obtained using this protocol without the need of growth factors. We also show that the differentiated GABAergic neurons produce large outward K^+ currents typical of mature neurons. Our results shed a new light on the effect of cell environment during the neuronal differentiation of mouse ES cells and may provide new therapeutic opportunities for the future treatment of neurological diseases.

2. Materials and methods

2.1. ES cell culture

The cells used in this study were the EMC-ES-Hoxa1-1 (E1) and JI Wild type mouse ES cell-lines. The generation and characterization of the E1 ES cell line from C57Bl/6 mice was previously described (Martinez-Ceballos et al., 2005). J1 is a commonly-used ES cell line obtained from the American Type Culture Collection (ATCC). The ES cells were maintained in an undifferentiated state by culturing them in ES medium (Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% ES qualified fetal bovine serum (Atlanta Biologicals), 100 μM MEM nonessential Amino acids, 0.1 mM β-mercaptoethanol, Penicillin and Streptomycin (Invitrogen), 1 mM sodium pyruvate, and 1×10^3 U/ml leukemia inhibitory factor (LIF, Esgro, Millipore)). To avoid spontaneous differentiation, the ES cells were passaged every two days for up to three passages before induction of differentiation.

2.2. Generation of embryoid bodies

To determine the percentage of neurons obtained using a standard protocol that requires EB formation, 3×10^6 cells were plated onto 100 mm non-adherent dishes in the absence of LIF as previously described (Martinez-Ceballos and Gudas, 2008). The aggregates were cultured in ES medium for 8 day and the medium was changed every two days. RA (5 μM, 5RA) or vehicle-only (ethanol, 0RA) was added on days 4 and 6 of EB formation. On day 8, the EBs were harvested, disaggregated with Accutase (Millipore), and cell viability was determined by the trypan blue exclusion method using a Cellometer (Nexcellom, Inc.). In separate experiments, day 8 EBs were either fixed for sectioning as previously described (Martinez-Ceballos and Gudas, 2008) or disaggregated and fixed for immunofluorescence analyses.

2.3. ES cell encapsulation

Undifferentiated ES cells were suspended in a solution of sterile 1.1% (w/v) alginic acid and 0.1% (v/v) porcine gelatin at a final concentration of 2.5×10^4 cells/ml. Using an 18-gauge (18 G) needle, the cell suspension was dropped into a sterile alginate gelation solution (100 mM $CaCl_2$, 10 mM HEPES, 0.01% (v/v) Tween 20) at pH 7.4 with stirring as described by Hwang et al. (2009). The average volume of the cell suspension drops was 50 μl. The hydrogels were allowed to solidify in the $CaCl_2$ solution for 5 min and were washed 3 times with ES medium. The encapsulated ES cells were then cultured in ES medium without LIF for 8 days. Medium was replenished every 2 days and RA (5 μM, 5RA) was added at days 4 and 6. Control cells in hydrogels were treated with vehicle only (0RA). On day 8, depolymerization buffer (50 mM tri-sodium citrate dihydrate, 77 mM sodium chloride and 10 mM HEPES) was added to the hydrogels to harvest the cells for the appropriate assays.

2.4. Neuronal differentiation

To induce neuronal differentiation, both day 8 encapsulated and non-encapsulated cells were harvested and plated at a density of 1.5×10^5 cells/cm^2 on tissue culture dishes pre-coated with poly-D-lysine and laminin (PDL/laminin) as described by Bibel et al. (2004). The cells were cultured in N2 medium (Neurobasal medium containing N2 supplement (Invitrogen)) to allow for the selection of neuronal lineages. The N2 medium was changed 2 h after plating and then again after 24 h. The total culture time in N2 medium was 2 days (2N, Bibel et al., 2004) In order to promote neuronal maturation, the N2 medium was removed and cells were cultured for four days in maturation medium (B27-supplemented Neurobasal medium) to induce neuronal maturation (4 M stage). For the first two days of this maturation step, 5 μM RA was also added to the cells. For the remaining two days of this 4 M stage, cells were cultured in the absence of RA. The cell fate of the differentiated cells obtained at 4 M was examined by immunofluorescence.

2.5. Immunofluorescence analyses

ES cells were fixed in 4% formalin for 15 min, followed by permeabilization for 20 min in 0.1% Triton X-100. Samples were blocked with goat or horse serum and incubated with the appropriate primary antibodies for 1 h. The primary antibodies used include rabbit anti- β-tubulin III (Covance, Berkeley, CA), mouse anti-Nestin (Rat401, Developmental Studies Hybridoma Bank, Iowa City, IA), guinea pig anti-GABA (Millipore), rabbit anti-GAD 65/67 (Millipore), goat anti-Parvalbumin (Santa Cruz Biotechnologies), and goat anti-Somatostatin (Santa Cruz Biotechnologies). All primary