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Research Article

Critical roles of Src family tyrosine kinases in excitatory neuronal differentiation of cultured embryonic stem cells

Michelle Hedrick Theus[a,b], *Ling Wei*[a], *Kevin Francis*[a], *Shan Ping Yu*[a,b,*]

[a]*Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425, USA*
[b]*Department of Pharmaceutical Sciences, Medical University of South Carolina, Charleston, SC 29425, USA*

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ABSTRACT

Embryonic stem (ES) cells have been tested for potential cell transplantation therapy for CNS disorders. Understanding their differentiation mechanism and identifying factors involved in driving excitatory and inhibitory neuron lineages should enhance the efficacy and efficiency of the cell transplantation therapy. We tested the hypothesis that selective expression of Src family tyrosine kinases is required for phenotype-specific differentiation and functional maturation of ES cell derived neurons. Cultured mouse pluripotent ES cells were treated with retinoic acid (RA) to induce neural differentiation. After RA induction, neurons derived from ES cells showed significant neurite growth, increased expression of Src, Fyn and Lck and an extension of Src kinase expression from cell body to neurite processes. ES cell derived neuron-like cells expressed neurofilament, synaptophysin, glutamate receptors, NMDA and kainate currents, became vulnerable to excitotoxicity and formed functional excitatory synapses. These developmental events were blocked or attenuated when cells were grown in the presence of Src family kinase inhibitor PP2. However, there was no change in the expression of GABAergic-specific protein GAD67 during PP2 treatment. Our data suggest that Src tyrosine kinases are involved in the terminal differentiation of excitatory neuronal phenotype during ES cell neural differentiation after RA induction.

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Introduction

Embryonic stem (ES) cells have provided a valuable resource for potential cell transplantation therapy for the treatment of cerebral ischemia, spinal cord injury and neurodegenerative diseases [1–6]. ES cells may differentiate into multiple cell types. Neural-induced stem cells, in particular, are capable of differentiating into neuronal lineage cells [1–3]. Cultured neural-derived ES cells have been shown to express multiple genes coding for proteins associated with basic neuronal properties. These cells express functional receptors for a variety of neurotransmitters and major ionic channels [1,4]. After transplantation, ES-cell-derived neural precursors can incorporate into the CNS and differentiate into neurons and glial cells [5–10]; however, regulatory factors involved in cell fate choices, particularly subclasses of neurons, are unclear.

* *Corresponding author*. Department of Pharmaceutical Sciences, 280 Calhoun Street, Medical University of South Carolina, Charleston, SC 29425, USA. Fax: +1 843 792 1712.
E-mail address: yusp@musc.edu (S. Ping Yu).

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Since neuronal differentiation is a key step in ES cell transplantation therapy, understanding the mechanisms that control or affect this process will benefit further investigations and potential stem cell therapy. Neuronal induction by retinoic acid (RA) is commonly used in ES cell differentiation. Upon differentiation, RA-treated cells give rise to defined and developmentally restricted neuronal lineages [11,12]. For example, neurons derived from RA-treated ES cells typically develop into glutamatergic and GABAergic subtypes of neurons [1,13,14]. A better understanding of the mechanism that controls ES cell fate toward different neuronal phenotypes becomes an important issue in ES cell transplantation therapy.

Clues to identifying the differentiation pathways leading to the generation of specific fully functional subclasses of neurons after RA induction of ES cells can be unveiled by studying embryonic neural development. Furthermore, it may be possible to recapitulate this development in a dish by altering cell culture conditions during ES cell differentiation in order to generate the specific neuronal phenotypes required for cell replacement therapy in a desired region of the damaged CNS. Src family tyrosine kinases are developmentally regulated genes in the CNS [15–18]. For example, the increase in src activity in the striatum and hippocampus regions of the developing brain coincides with the peak periods of neurogenesis and neuronal growth. In addition, the levels of pp60c-src activity are much higher throughout the embryonic brain than those observed in the adult. The expression of pp60c-src is not only developmentally regulated, but demonstrates a regionally distinct pattern of expression [19]. In the cortex, src activity steadily rises during gestation, while in the basal forebrain and midbrain, maximal activity is observed at E17 then declines to adult levels. The Src kinase is the leading protein of at least nine family members, five of which, Src, Fyn, Lck, Lyn, and Yes, are expressed in the CNS [20–22] and are known to be involved in processes such as axonal outgrowth and stabilization, ion channel regulation, excitatory synaptic transmission and plasticity in the adult brain [23–25].

The glutamatergic synapse is a fundamental element in excitatory neurotransmission [26–29]. More specifically, the NMDA subtype of glutamate receptors plays a central role in neuronal development [30], synaptic plasticity [31] and excitotoxicity [5]. The activity of the NMDA receptor is regulated by Src family kinases [32,33]. We tested the hypothesis that the developmental potential of glutamatergic neurons derived from mouse embryonic stem cells following RA induction may require activation of specific src family members during discrete stages of differentiation. The term “differentiation” is a broad concept that may include “early”, “intermediate” and “terminal” differentiation [13]; in this report, the process from neural progenitor cells to mature neurons is regarded as part of neuronal differentiation, i.e. terminal differentiation. In the present study, we explored a specific regulatory role of src kinases in excitatory neuronal differentiation of ES cells after RA neural induction, specifically in the terminal differentiation of fully functional glutamatergic neurons and in proper formation of excitatory synapses in vitro.

Materials and methods

Cell culture preparation

ES cell cultures were prepared from stocks of the D3 ES cell line (from Dr. D. Gottlieb, Washington University in St. Louis, MO, USA) maintained in our laboratory. ES cells of no more than 20 passages were used for experiments. The passage procedure of undifferentiated ES cells was performed every 2 days on Gelatin-coated T25 flasks in the presence of 1000 units/ml of leukemia inhibitory factor (Chemicon, Temecula, CA, USA) and 0.1 mM β-mercaptoethanol in the induction media (ESIM). ESIM consists of Dulbecco’s modified eagle medium with L-glutamine (Gibco, Carlsbad, CA, USA), without pyruvate, supplemented with 10% fetal bovine serum, 10% newborn calf serum, 8 μg/ml guanosine, 7.3 μg/ml cytidine, 7.3 μg/ml uridine and 2.4 μg/ml thymidine.

Neural lineage induction was completed by exposing differentiating embryoid bodies to RA, known as the 4–/4+ RA protocol [1,6]. Briefly, ES cells were harvested from T25 flasks by trypsinization with 0.25% trypsin and placed into a standard 100-mm bacterial Petri dish containing ESIM free of LIF or β-mercaptoethanol. Four days later, the medium was replaced with fresh ESIM containing 5×10^{-7} M all-*trans* RA (R-2625; Sigma, St. Louis, MO, USA) and incubated for four more days.

Following the 4–/4+ RA treatment, embryoid bodies were washed in a balanced salt solution and dissociated with 0.25% trypsin–EDTA for 10 min at 37°C. ESIM was added to stop trypsinization and cells were triturated into single-cell suspension and centrifuged at $1000\times g$ for 5 min. Medium was removed and cells were resuspended in modified Sato medium [5,6]. Cells were then plated on poly-D-lysine and laminin (PDL) coated 35-mm glass-bottom dishes, 24-well plates or 35-mm dishes with a preexisting astrocyte monolayer prepared from neonatal mouse cortex [34]. In tyrosine kinase inhibition experiments, the tyrosine kinase inhibitor PP2 (Calbiochem, San Diego, CA, USA) was initially suspended in DMSO (0.1% of DMSO). It was then added into the medium of cultured ES cells at a final concentration of 10 μM. Media was replaced every 2 days by media containing fresh PP2 to prevent degradation of the inhibitor. Cultured neuronal cells survive much better and longer when cultured with glial cells. To reduce the basal cell death that might occur when cultured on poly-D-lysine-/PDL-coated dishes and to exclude the possibility that PP2 might induce cell death, long-term experiments (≥5 days) and experiments with PP2 were performed using mixed cultures of ES cells on a glial bed. Control experiments were then carried out using the same mixed cultures.

Protein isolation and SDS-PAGE analysis

Differentiating ES cells were analyzed for Src family tyrosine kinase expression after 4–12 days in culture. Cells were washed twice with ice-cold PBS and lysed in a medium containing 50 mM Tris–HCl (pH 8.8), 150 mM NaCl, 2 mM EDTA, 1% SDS, 2 mM sodium orthovanadate, 1% NP-40, 1% sodium deoxycholate, leupeptin, aprotinin, pepstatin, phosphates inhibitor and PMSF. Cells were scraped from the dish,

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