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Development of membrane ion channels during neural differentiation from human embryonic stem cells

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ABSTRACT

Objective: For human embryonic stem cells (hESCs) to differentiate into neurons, enormous changes has to occur leading to trigger action potential and neurotransmitter release. We attempt to determine the changes in expression of voltage gated channels (VGCs) and their electrophysiological properties during neural differentiation.

Materials and methods: The relative expressions of α-subunit of voltage gated potassium, sodium and calcium channels were characterized by qRT-PCR technique. Patch clamp recording was performed to characterize the electrophysiological properties of hESCs during their differentiation into neuron-like cells.

Results: Relative expression of α-subunit of channels changed significantly. 4-AP and TEA sensitive outward currents were observed in all stages, although TEA sensitive currents were recorded once in rosette structure. Nifedipine and QX314 sensitive inward currents were recorded only in neuron-like cells.

Conclusion: K^+ currents were recorded in hESCs and rosette structure cells. Inward currents, sensitive to Nifedipine and QX314, were recorded in neuron-like cells.

1. Introduction

Human embryonic stem cells (hESCs) are derived from the inner cell mass of pre-implantation embryos and maintain the developmental potency of embryonic originator cells. These cells are characterized by special gene profile, the capacity to either proliferate indefinitely and the potential to originate tissue-specific progenitors or differentiated cells [1]. One of the well-studied differentiation from hESCs is neural differentiation in which selective conditions have plenty of similarities to the native development experiences.

Development of neurons, which are the fundamental cells of nervous system, is an orchestrated process in which cellular behavior is configured toward nerve signal transmission. This is initiated whenever bone morphogenic protein (BMP) is antagonized by other environmental factors, like Chordin and Noggin for neuroectoderm commitment. Subsequently, as a result of morphogenesis, neural plate transforms into neural-tube that consists of multipotent neural stem cells ready to differentiate into neurons and glia. Further neurogenesis is caused by consistent secretion of sonic hedgehog from underneath mesoderm [2]. This process can be imitated *in vitro* and then, generated cells should be characterized in different aspects, namely gene expression, protein products and membrane characteristics, especially voltage gated ion channels and their function.

Specifically, electrophysiological properties of cells during their differentiation are one of the key parameters in distinguishing an individual cell from others and this task is done by patch clamp technique [3]. Ion channels play a critical role in cell excitability and cellular communication. For instance, ion channels in neuronal,

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myoblastic and pancreatic cells can produce action potential profiles and subsequent physiological functions such as transmission of messages between nerve cells, heart beat and insulin secretion [4,5]. The role of ion channels in unexcitable cells is also considerable; many types of K^+ channels have crucial roles in the proliferation, cell cycle and apoptosis of mesenchymal stem cells, human and mouse pluripotent stem cells, neural stem cells and tumor cells [3,6–10].

Also, numerous researchers have introduced several functional ion channels in human and mouse ESCs [9]. They represented that inhibition of specific K^+ channels can affect the proliferation of hESCs. Application of voltage gated K^+ channel blockers significantly reduced the proliferation of mouse ESCs and hESCs in a dose-dependent manner [9].

The functional properties of neuron-like cells derived from hESCs are largely unknown. As pointed out by several studies, 4-AP efficiently blocked the A-type K^+ channels (I_A) and also non-selectively inhibited the delayed rectifier K^+ channels (IK_{DR}) in human neural stem cells. In addition, the expression pattern of ion channels in differentiated neural cells derived from human neural stem cells was characterized and it was reported that ion channel expression pattern changed during differentiation progress [10]. It also has been shown IK_{DR} and the inward rectifier K^+ channels are expressed in human neural stem cells. Therefore, these two types of K^+ channels could represent physiological function of neural stem cells [11].

In this study hESCs were differentiated into neuron-like cells. Afterward, the expression of α-subunit of ion channels and their currents in addition to membrane passive properties were investigated.

2. Material and methods

2.1. Human embryonic stem cell culture and neural induction

hESCs (Royan H6 cell line) seeded on matrygel (E1270; Sigma) substrates and cultured in Dulbecco's Modified Eagle's Medium (DMEM, 21331–020; Gibco, UK) supplemented with 20% knock-out serum replacement (KOSR; 10828–028; Gibco), 1% nonessential amino acid (11140–035; Gibco), 2 mM L-glutamine (25030–024; Gibco), 0.1 mM β-mercaptoethanol (M7522; Sigma, St Louis, MO, USA) 100 U/mL penicillin-streptomycin (15070–063; Gibco) and 100 ng/mL basic-fibroblast growth factor (bFGF; F0291; Sigma) [12].

hESCs induce to form rosette structures in the presence of retinoic acid (RA; 2 mM; R2625; Sigma), bFGF (20 ng/mL, F0291-Sigma), Noggin (500 ng/mL, 6057-NG, R&D) and N2 supplement (175020–01, Sigma). Further maturation to neuron-like cells occurred on laminin (5 mg/mL; L2020; Sigma) and poly-L-ornithine (15 mg/mL; P4957; Sigma) coated plates in neurobasal medium (21103049-Sigma) containing shh (50 ng/mL; 1314-SH; R&D), 200 mM ascorbic acid (AA; A8960; Sigma), retinoic acid, 2% N2 supplement (17502–048; Gibco), and 2% B27 (17504–044; Gibco) (Fig. 1A) [13].

2.2. Immunofluorescence staining

Immunofluorescence staining were done as previously described [12,13]. In brief, the cells were fixed with 4% paraformaldehyde and non-specific binding was blocked with 10% normal goat serum. Samples incubated with primary antibody for 60 min at 37 °C. At the end of the incubation time, they were rinsed with PBS and incubated with the fluorescence isothiocyanate (FITC)-conjugated anti-mouse IgG (1:100; Sigma). Then the cells were analyzed under fluorescent microscope (Nikon, Tokyo, Japan).

2.3. Quantitative real-time RT-PCR

Total RNA was extracted from the hESCs (stage 0), rosette structures (stage 1), neural tube formations (stage 2) and neural-like cells (stage 3) by TRIZOL (Cinnagen, RN7713C). RNA samples were digested with DNaseI (Fermentas; EN0521). Then, standard cDNA synthesis was performed using 1 μg total RNA, oligo (dT) and the RevertAidTM Minus First Strand cDNA Synthesis Kit (Fermentas; K1622) according to the manufacturer's instructions. Relative gene expression was analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$). Target genes were normalized to reference gene, β-Actin and calibrated for each sample against hESCs.

2.4. Whole cell patch clamp recording

Electrophysiological properties of hESCs during neural differentiation investigated by performing whole-cell patch clamp technique. Bath solution contained (mM): NaCl 140, KCl 4.5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, and D-Glucose 10 (PH = 7.4). The pipette solution contains (mM): KCl 140, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, and EGTA 11 (PH = 7.2–7.4). The bath solution for recording calcium and sodium currents contained (mM): NaCl 160, $CaCl_2$ 2, HEPES 10 and D-glucose 10 (PH = 7.4) and in internal solution $CsCl_2$ 130 mM substituted for KCl and $CaCl_2$. We applied QX-314 (Santa Cruz, SC-3579) to the pipette solution for blocking Na^+ channels. TEA (MERCK, S31340-036) 10 mM as a blocker of IK_{DR} and 4-Aminopyridin (Sigma, A7, 840–3) as a blocker of I_A were added to the bath solution. All experiments carried out at room temperature 22 °C. Electrode tip (Harward Apparatus, GC150F-10) resistance in bath solution was about 2–5 MΩ. Data were low-pass filtered at 3 kHz and acquired at 2 kHz with a Multiclamp 700B amplifier equipped with a Digidata 1440 A/D converter (Molecular Devices, USA). The signal was recorded on a PC for offline analysis via Axon pClamp-10 acquisition software. All data analyzed by Clampfit10 software (Axon instrument, USA).

3. Statistical analysis

The results were expressed as means ± SEM. Mean differences were analyzed using one-way ANOVA and Tukey posttest. $P < 0.05$ was considered statistically significant.

4. Results

4.1. In vitro differentiation of hESCs into neuron-like cells

hESCs were provided in Royan institute cell bank and this colony forming and pluripotent cells were differentiation onset as “stage 0” (Fig. 1A). Following retinoic acid and Noggin treatment in “stage 1”, columnar cells appeared, reminding of rosette structures (Fig. 1B) in which SOX1, PAX6 and NESTIN markers were detectable as the first sign of neural determination (Fig. 1F). Subsequently, Noggin elimination and bFGF enhancement together reshape columnar cells to denser cell aggregates named neural-tube (Fig. 1C). Acquired neural-tube cells were also positive for SOX1, PAX6 and NESTIN (stage 2, Fig. 1 G). Henceforth, neural-tube structures were isolated manually and suspended for 6 days (Fig. 1D). After a 6 days interval, suspended neural tubes transfer on a laminin/poly-L-ornithine substrate. Within a few days of adherent culture, numerous processes grew out from the neural-tube giving the appearance of starburst. Soon, fiber bundles emanated from neural-tube and a few small migrating cells were seen in close association with the fibers. Neuron-like cells were achieved with more apparent ramified fibers in 12 days (Fig. 1E). Accordingly, MAP2 and TUJ1 positive early-matured neurons were obtained (Fig. 1H).

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