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# Differential Expressions of Gap Junction Proteins during Differentiation of Rat Neuronal Stem Cells

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*ABSTRACT* : **Gap junctional intercellular communication (GJIC) plays a key role during development, process of tissue differentiation, and in maintenance of adult tissue homeostasis. Neural stem cells leading to formation of cell clusters termed "neurospheres", can differentiate into neurons, oligodendrocytes, and astrocytes. We investigated the expression levels and distribution of connexin43 (Cx43) and connexin32 (Cx32), abundant gap junctional protein in neural cells and in neurospheres isolated from rat fetus embryonic day (ED) 17. During differentiation of neurospheres, expression of Cx43 and 32 were increased time-dependently within 72 h, and then decreased at 7 day in western blot analysis. TPA-induced inhibition of GJIC was confirmed by decreased fluorescence by SL/DT assay, and induced hyperphosphorylation of Cx43 while no changes in Cx32 levels in western blot assay. Our results indicate that GJIC may be a crucial role in the differentiation of neuronal stem cell. And this GJIC can be inhibited by TPA through the hyperphosphorylation of Cx43.**

Keywords : **Neurosphere, Stem cells, gap junction, Cx43, Cx32, differentiation**

## Introduction

A great attention has been recently focused on neural stem cells (NSCs) leading to formation of cell clusters termed "neurosphere" because of their therapeutic potential (Livesey and Cepko, 2001). These cells maintain the capacity to produce the three main mature cell classes of the central nervous system: neurons, oligodendrocytes, and astrocytes (Temple, 2001). There is growing evidence indicating that passage of inos, metabolites, and second messengers between cells via connexin-mediated gap junction alters neural progenitor fate (Bittman *et al.*, 1997).

Gap junction channels are conduits for the direct cell-to-cell exchange of small molecules and ions (Simon and Goodenough, 1998). This is known as gap junctional intercellular communication (GJIC) and functions in electrical coupling, homeostasis, growth regulation, cell coordination, and many other physiological process. Reduced GJIC has been associated with several human diseases that include cancer, teratogenesis, cataracts, hereditary deafness, and peripheral neuropathy (Ruch *et al.*, 2001; Cho *et al.*, 2002; Kang *et al.*, 2002). Gap junction channels are formed by protein subunits known as connexins. At least 13 mammalian connexins exist and are expressed in a cell- and tissue-specific manner (Simon and Goodenough, 1998). During past decades, several studies have demonstrated the existence of gap junctions in specific areas of the mammalian brain (Dermietzel *et al.*, 1989). In adult brain, astrocytes are abundantly interconnected by gap junctions containing Cx43, whereas oligodendrocytes express Cx32. Cx43 and Cx26 are also found in meningeal and ependymal cells (Cicirata *et al.*, 1998).

The aim of this study was to investigate the expression of GJIC during development of neurospheres, containing stem cell enriched population.

## Materials and Methods

### Primary rat brain culture

The Pregnant Sprague-Dawly rat (Bio Genemics, Inc., Seoul, Korea) were sacrificed at embryonic day (ED) 17 (Kang, *et al*, 2001), embryos placed in a petri dish containing Hanks balanced salt solutions (HBSS, Gibco, USA). For dissociation and plating cells, the cortex was dissected from the rest of the brain and isolated cortex

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transferred to a 0.5% trypsin solution (Gibco, USA). To obtain small clumps of cells the solution was gently pipetted up and down about 20 times in 5 ml pipette until it attained a milky, homogeneous appearance. The suspension was incubated for 30 min at 37°C. There after 1 ml of PBS containing 0.04% deoxyribonuclease (DNase, type I, 650 KU/mg, Sigma, USA) was added to the tissue. The soultion was pipetted up and down several times. Cells were plated in 100 mm dished (Nunc, Denmark) and maintained in a 1:1 mixture of Dulbeco's Modified Eagle Medium and F12 medium (DMEM/F12, Gibco, USA) supplemented with 5% fetal bovine serum (Gibco, USA), basic fibroblast growth factor (bFGF, Boehringer mannheim, USA), Epidermal growth factor (EGF). Dishes were incubated ar 37°C in humidified 5% $CO_2$: 95% air.

### MTT assay

Cytotoxicity was determined by MTT assay and cells were treated with TPA for 24 hr. Follwing chemical treatment, the cells were rinsed three times with PBS and then incubated for 5 hours before the end of the incubation add 200 ul of MTT. After media was removed, added 200ul of DMSO to each well and then pipetted up and down to dissolve the crystals. We determined absorbance at 540 nm using an ELISA reader (EL800, Bio-Tek Instruments, Inc, Vermont, USA).

### Scrape Loading Dye Transfer (SL/DT) assay

The scrape loading/dye transfer (SL/DT) technique was adapted using the method of EL-Fouly *et al.* Cell were treated with TPA for 1 h. The GJIC assay was conducted at non-cytotoxic dose levels of the samples, as determined by the MTT assay. Following incubation, the cells were washed twice with 2 ml of PBS. Lucifer yellow(Sigma, USA) was added to the washed cells and three scrapes were made with a surgical steel-bladed scalpel at low light intensity. These three scrapes were made to ensure that the scrape traversed a large group of confluent cells. After a 3 min incubation period the cells were washed with 10 ml of PBS and then fixed with 2 ml of 4% formalin solution. The distance traveled by the dye in a direction perpendicular to the scrape was observed with an inverted fluorescent microscope (Olympus Ix70, Okaya, Japan).

### Western Blot analysis

Cells were grown in a 100 mm tissue culture dish (Nunc, Rochester, NU, USA) to the same confluency and were then treated with test compounds. Proteins were extracted with 20% SDS solution containing 1 mM phenylmethylsulfonyl fluoride (a pretease inhibitor), 10 mM iodoacetoamide, 1 mM leupeptin, 1 mM antipain, 0.1 mM codium orthovanadate and 5 mM sodium fluoride.

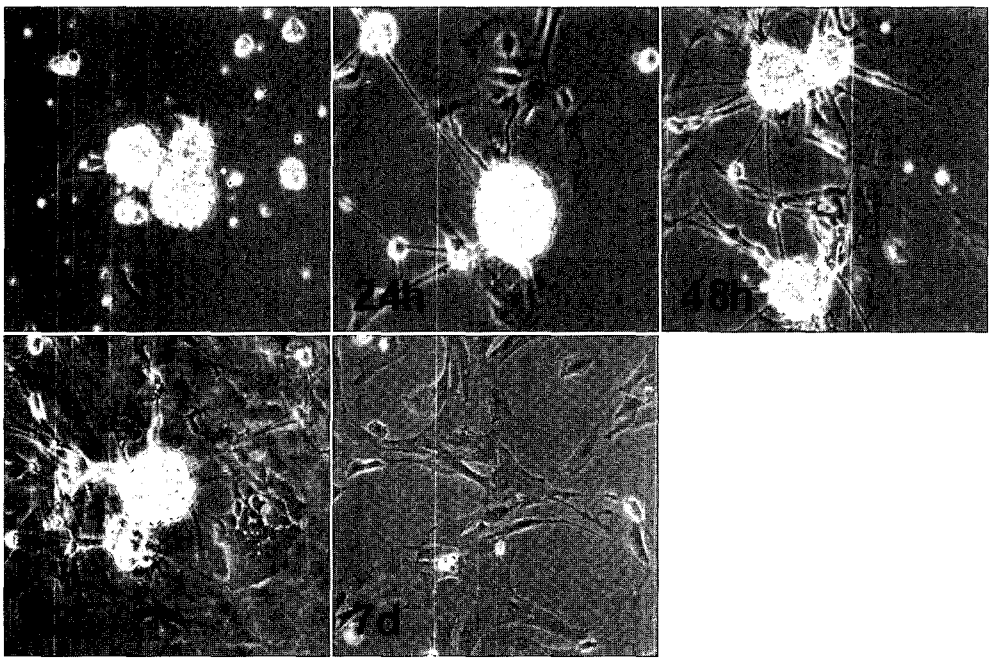


**Fig. 1.** Differentiation of neurospheres and neurosphere-derived cells (NS; neurosphere).

Protein content was determined using the DC assay kit (Bio-Rad, Hercules, USA), and separated on 12.5% SDS-PAGE according to the method of Ladmmli. They were then transferred to nitrocellulose membranes at 100V, 350 mA for 1 h. mouse monoclonal anti-connexin 32, 43 (Chemicon, USA) were used according to the manufacturer's instrusctions and protein bands were detected using an ECL detection kit (Amersham, USA).

## Results

### Morphologies and GJIC expression in neurospheres and neurosphere-derived cells

Cells derived from the ED17 rat brain were grown in the presence of bFGF, EGF, and FBS leading to the formation of neurospheres. Neurospheres differentiated until 72 h continuously, neurospheres disappeared after passage (Fig. 1). To investigate the expression of GJIC on neurosphere and neurosphere-derived cells during differentiation, we examined Cx32 and Cx43 protein expression by western blot analysis. Cx43 and Cx32 protein were increase in a time-dependant manner within 72 h, and then decreased at 7d. Neurosphere-derived cells were increased time-dependently within 72 h, but decreased at 7 d, regardless no change of levels of β-actin (Fig. 3).

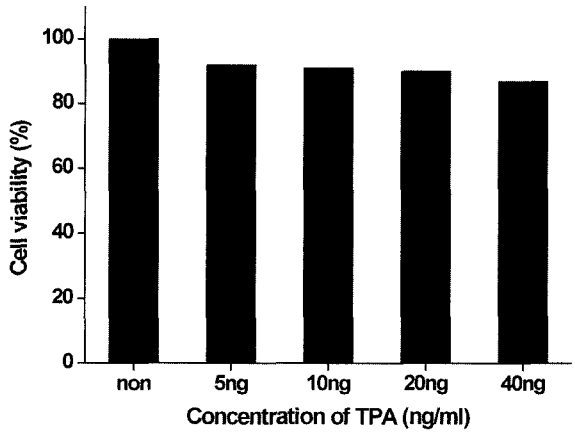


**Fig. 2.** Cytotoxicity of TPA.

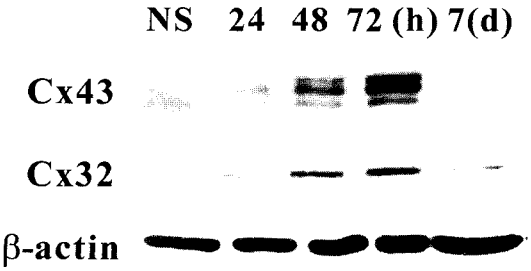


**Fig. 3.** Time-course study of GJIC expression of Cx43 and 32 in western blot analysis.

### Effect on GJIC treated neurosphere-derived cells with TPA

To determine the appropriated dose of TPA, its cytotoxicities to neurosphere-derived cells were assessed using the MTT assay. No cytotoxic effects were observed on cells treated with all dose of TPA (0, 5, 10, 20, and 40 ng/ml) (Fig. 2). We next determined the ability of

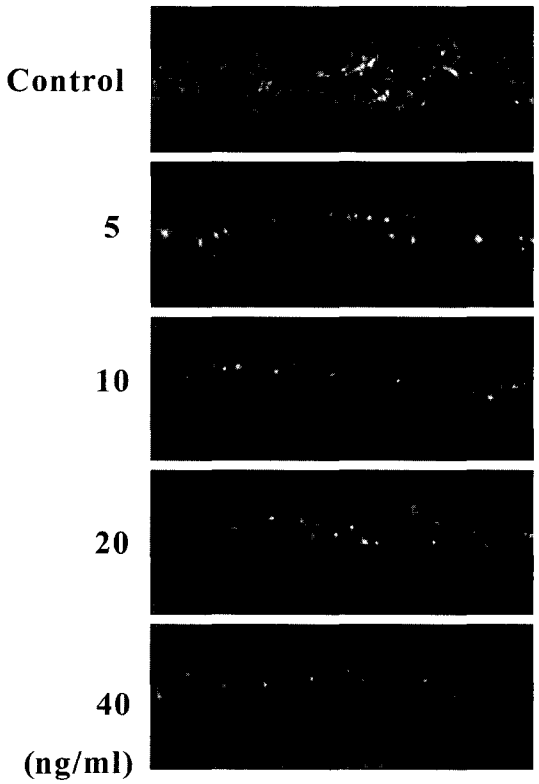


**Fig. 4.** GJIC expression on neurosphere-derived cells treated with TPA using SL/DT.

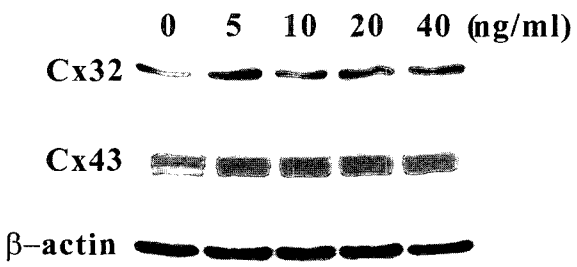


**Fig 5.** Expression of Cx43 and 32 on neurosphere-derived cells treated with TPA in western blot analysis.

neurosphere-derived cells to communicate through gap junctions under TPA exposure, GJIC assay was performed using SL/DT. Treatment with TPA for 1h significantly decreased the extent of GJIC of cells compared with the GJIC of control (Fig. 4). In western blot analysis, neurosphere-derived cells treated with TPA expressed hyperphosphorylation of Cx43, but TPA had no effect on Cx32 (Fig. 5).

## Discussion

In this study, all neurospheres didn't show any expression of Cx43 and 32, however, the expression of Cx43 and 32 were increased progressively until 72 h, and then decreased at 7d in western blot analysis. There is an evidence neurospheres as stem cell clusters. *In vitro*, pharmacological inhibition of GJIC decreases the percentage of embryonic progenitors that enter S-phase and inhibits terminal differentiation of embryonic carcinoma cells into neurons and glia (Bani-Yaghoub *et al.*, 1999). Previous studies have shown that hippocampal progenitor cells are highly coupled by gap junction composed of Cx43 during early ontogeny, before the functional expression of chemoresponsiveness to a variety of developmentally mediated neurotransmitters (Rozental *et al.*, 1995). Because neuroblast progressively differentiate, Cx43 expression was decreased (Rozental *et al.*, 1998). Therefore, expression of Cxs is related to differentiation into other neural cells and proliferation.

TPA almost immediately disrupted GJIC in SL/DT assay and induced hyperphosphorylation of Cx43, but not reduced Cx32 content in western blot analysis. Soroceanu *et al* showed a marked decrease in GJIC in human grade IV astrocytomas and present correlative findings regarding expression levels of connexins and the mitotic activity in acute biopsies from the same patients. A correlation between the loss of dye-coupling, Cx43 hyperphosphorylation, and gap junction internalization was observed in WB-F344 cells treated with TPA (Ruch *et al.*, 2001). It is unknown whether these processes are interdependent. However, loss of dye-coupling can be occured in the absence of Cx43 hyperphosphorylation in TPA-treated cells (Husoy et al., 1993).

Documenting the extent of GJIC is important for understanding the molecular basis of various CNS disorders, as well as for designing approaches aimed at restoring intercellular communication and rendering malignant gliomas more amenable to gene therapy (Yamasaki *et al.* 1999). These data suggest that expression of GJIC could be an important role to help define biological function during differentiation of neurospheres which containing stem-cells enriched population.

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