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Noggin *versus* basic fibroblast growth factor on the differentiation of human embryonic stem cells

Yan Zhang[1], Junmei Zhou[2], Zhenfu Fang[1], Manxi Jiang[1], Xuejin Chen[1]

1 *Department of Laboratory Animal Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China*
2 *Tissue Engineering Laboratory, Shanghai Institute for Pediatric Research, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200092, China*

Research Highlights

(1) The present study is the first to analyze the difference of Noggin and basic fibroblast growth factor to induce neural precursor differentiation of human embryonic stem cells.
(2) Using a monolayer differentiation method, the differentiation effects of Noggin and basic fibroblast growth factor were compared. Noggin was more effective than basic fibroblast growth factor in the differentiation of neural precursor cells.

Abstract

The difference between Noggin and basic fibroblast growth factor for the neural precursor differentiation from human embryonic stem cells has not been studied. In this study, 100 μg/L Noggin or 20 μg/L basic fibroblast growth factor in serum-free neural induction medium was used to differentiate human embryonic stem cells H14 into neural precursors using monolayer differentiation. Two weeks after induction, significantly higher numbers of neural rosettes formed in the Noggin-induced group than the basic fibroblast growth factor-induced group, as detected by phase contrast microscope. Immunofluorescence staining revealed expression levels of Nestin, β-III Tubulin and Sox-1 were higher in the induced cells and reverse-transcription PCR showed induced cells expressed Nestin, Sox-1 and Neurofilament mRNA. Protein and mRNA expression in the Noggin-induced group was increased compared with the basic fibroblast growth factor-induced group. Noggin has a greater effect than basic fibroblast growth factor on the induction of human embryonic stem cell differentiation into neural precursors by monolayer differentiation, as Noggin accelerates and increases the differentiation of neural precursors.

Key Words

neural regeneration; stem cells; basic fibroblast growth factor; Noggin; human embryonic stem cells; neural precursors; neural differentiation; grants-supported paper; neuroregeneration

INTRODUCTION

Neural precursors induced from human embryonic stem cells *in vitro* can supply highly qualified seed cells for cellular therapy to treat nervous system diseases, such as Parkinson's disease, Alzheimer's disease and nerve damage[1]. Recently, differentiation of human embryonic stem cells into neural precursors has become the focus of medical studies. Human embryonic stem cells are characterized by their capacity for self-renewal and potential to differentiate into cells of all three germ layers including functional neural line cells, which can be transplanted into an injured vertebrate central nervous system and integrate with host

Yan Zhang, Master.

Corresponding author: Xuejin Chen, Ph.D., Professor, Department of Laboratory Animal Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China, chenxuej@yahoo.com.cn.

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cells to restore destroyed neural tissues[2].

Increasing evidence has demonstrated the directed neural differentiation of embryonic stem cells and methods of neural differentiation can generally be divided into three types: embryoid body-mediated, co-culture with feeder layers, and monolayer differentiation[3]. Embryoid body-mediated differentiation was the earliest and most conventional method[4]; however, it is easily contaminated, time-consuming, has a low efficiency, and the neural cells produced do not have stable maturity. Therefore, this technique is unlikely to meet the needs of clinical cell therapy. The co-culture method adapts feeder layers, which can secrete inducing factors capable of promoting neural differentiation of human embryonic stem cells[4]. The feeder cell line should be immortalized and it is extremely difficult to separate the feeder layer from induced neural cells, so the co-culture method is unsuitable for cell transplantation. Monolayer differentiation[5] uses exogenous factors to improve neural precursor formation *via* human embryonic stem cells, such as Noggin and basic fibroblast growth factor, through activation of the endogenous cyclic adenosine monophosphate circuit or disruption of apoptosis, to produce a higher proportion of neural precursors in a short time. Synchronization of induced cells is much easier using monolayer differentiation than using the classical embryoid body-mediated method[6]. Monolayer differentiation employs a chemically-defined neural induction medium, does not require animal serum, has a higher reproducibility and enables investigation of the molecular mechanisms and signal transduction processes during neural differentiation. This can provide insights into the development of embryonic nerve system and help to explore the role of critical regulatory genes during neural differentiation. Therefore, we used the monolayer differentiation method with two growth factors to differentiate human embryonic stem cells into neural precursors in a short period. This experiment used the newly established human embryonic stem cells H14 to investigate the neural induction efficiency of Noggin and basic fibroblast growth factor in monolayer differentiation, to provide a further understanding of the regulatory mechanisms during early embryonic neural development.

RESULTS

Morphology of human embryonic stem cells H14 and induced cells

Human embryonic stem cells were observed by phase contrast microscope before induction. Undifferentiated human embryonic stem cells formed a flat monolayer with clear boundaries, large nuclei, a high nuclear-to-cytoplasmic ratio and two or three clear nucleoli, which were strongly refractive (Figure 1A). During induction, all groups of cells proliferated slowly, except negative control cells, followed by cell renewal. At the early induction stages, the induced human embryonic stem cells became smaller and grew directionally. Noggin-induced cells displayed the most obvious changes, initially forming typical rosette-like structures with a columnar epithelial-like morphology after 8 days of induction (Figure 1B). Two weeks later, Noggin-induced cells developed a number of protruding filaments, parts of which overlapped or connected to form a network (Figure 1C). Changes in the basic fibroblast growth factor-induced cells occurred relatively slowly, with rosette structures first appearing on day 12 (Figure 1D). Compared with the Noggin-induced group, the rosette structures in the basic fibroblast growth factor-induced cells were smaller and less frequent (Figure 1E). No rosette-like structures emerged in the basic induction group (Figure 1F). All cells in the negative control group remained undifferentiated.

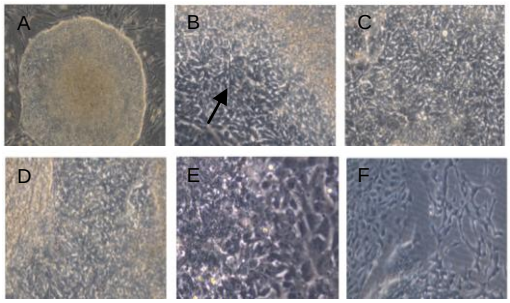

Figure 1 Morphology of induced cells at different stages of culture (phase contrast microscope, × 200).

(A) Human embryonic stem cells in the negative control group maintained an undifferentiated state.

(B) First appearance of typical neural rosette structures (arrow) in the Noggin-induced group on day 8.

(C) High quality rosette-like structures were observed in the Noggin-induced group on day 19.

(D) First appearance of neural rosette structures in the basic fibroblast growth factor-induced group on day 12.

(E) Morphology of the neural rosette structures at the last stage of induction in the basic fibroblast growth factor-induced group on day 21.

(F) No rosette-like structures were produced in the based-induced group.

Differentiated human embryonic stem cells H14 showed typical rosette-like structures

Neural precursors were generated *via* the direct induc-

tion method. In chemically-defined neural induction medium, the number of attached colonies generally increased as the border of the cells began to elongate. Two weeks later, a large number of mature neuroepithelial-like rosette structures were formed in the Noggin-induced and basic fibroblast growth factor-induced groups. Cells in the rosette-like structures were radically arranged and termed neural precursors. Cellular immunofluorescence staining revealed positive expression of Nestin in cells of the rosette-like structures. The neural rosette structures in the Noggin-induced group had a typically large cell, and a better formed structure than did the basic fibroblast growth factor-induced group (Figure 2). The induction efficiency was higher in the Noggin-induced group than the basic fibroblast growth factor-induced group ($P < 0.05$; Table 1).

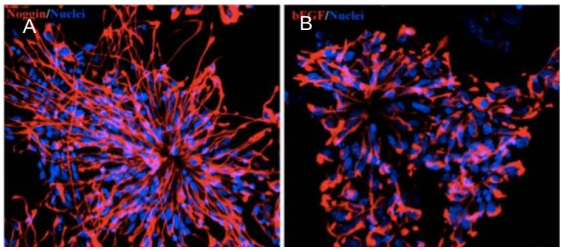

Figure 2 Neural rosette structures formed in the Noggin-induced and basic fibroblast growth factor-induced group (× 200).

Nestin immunoreactivity (red) in the neural rosette structures of the Noggin-induced group on day 19 (A) and basic fibroblast growth factor-induced group on day 21 (B) under confocal microscope. Nuclear staining with 4′,6-diamidino-2-phenylindole (blue). Fluorescence stain was Cy3.

Table 1 The number (/200-fold field) of neural rosette structures in the Noggin- and basic fibroblast growth factor (bFGF)-induced groups at 3 weeks after induction

Number of tests	Noggin-induced group	bFGF-induced group
1	16.40±2.80	8.70±2.87[a]
2	15.60±6.08	6.40±2.80[a]
3	13.30±2.67	7.20±2.78[a]

Data are expressed as mean ± SD. The experiments were repeated three times. Two-sample *t*-test was used to compare the number of rosette-like structures. [a]$P < 0.05$, *vs*. Noggin-induced group.

Identification of neural precursors

Immunohistochemical staining showed that, positive expression of Nestin, Sox-1, β-III Tubulin were observed in the neural rosette structures of both the Noggin-induced and basic fibroblast growth factor-induced groups (Figure 3), which were characteristic of neural precursors. Additionally, significantly greater numbers of neural precursors were formed by directed induction in the Noggin-induced group than the basic fibroblast growth factor-induced group ($P < 0.05$; Figure 4).

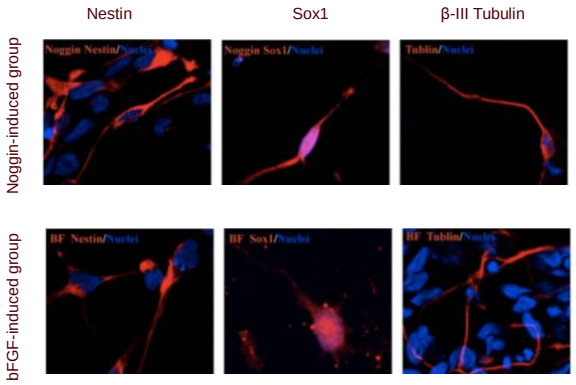

Figure 3 Immunocytochemical analysis of representative marker proteins including Nestin and Sox1 in Noggin- and basic fibroblast growth factor (bFGF)-induced neural precursors (× 200).

Positive expression of Nestin, nuclear expression of Sox1, and positive expression of β-III Tubulin in the Noggin-induced group.

Positive expression of Nestin, nuclear expression of Sox1, and positive expression of β-III Tubulin in the bFGF-induced group.

All cell markers are represented in red with 4′,6-diamidino-2-phenylindole nuclei staining (blue).

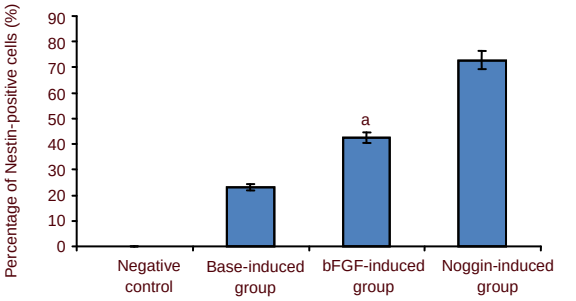

Figure 4 Efficiency of neural precursors formed by Noggin or basic fibroblast growth factor (bFGF) induction.

Nestin-positive cells are neural precursors. Data are expressed as mean ± SD. Two-sample *t*-test was used to analyze differences between any two groups. [a]$P < 0.05$, *vs*. Noggin-induced group.

Gene expression of neural precursors

The neural lineage-related gene neurofilament[7] was positively expressed in neural precursors (Figure 5). After 2 weeks of induction, the expression level of Nestin in neural precursors was high[4], consistent with observations that Nestin is specifically and highly expressed in neural precursors.

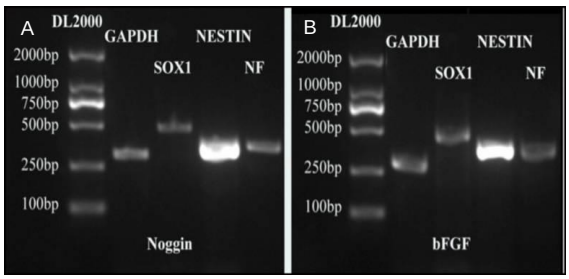

Figure 5 Reverse transcription-PCR analysis of gene expression in Noggin-induced (A) and basic fibroblast growth factor (bFGF)-induced (B) neural precursors.

The product sizes of Sox1, Nestin, neurofilament (NF) and GAPDH were 464, 368, 357 and 303 bp respectively. Sox1, Nestin and NF were positively expressed in induced cells. GAPDH was used as the reference gene.

DISCUSSION

During vertebrate nerve system development, ectoderm cells can spontaneously differentiate into neural cells without exogenous signals[8], and previous studies have shown that cell density is not a determining factor in cell differentiation[9-10]. The removal of a variety of inhibitory signals and contact inhibition between cells can induce undifferentiated human embryonic stem cells H14 to differentiate spontaneously to a neural lineage. The directed differentiation pathway was discovered on the basis of these observations. Smukler *et al* [11] cultured mouse embryonic stem cells at a density lower than 10 cells/μL in serum-free medium, feeder cells or growth factors, consistent with the default mechanism of directed differentiation. Corresponding studies using human embryonic stem cells supported the concept of a default neural differentiation mechanism in humans[12].

During embryonic development, neural differentiation can be inhibited by high levels of bone morphogenetic protein expression, which induces mesoderm cells and hinders H14 neuroepithelial differentiation. Noggin plays a critical role in embryo dorsal-ventral axis pattern formation, neural tube development and neural differentiation, and can prevent bone morphogenetic protein[3, 13] from binding homologous receptors on the ectoderm, therefore antagonizing bone morphogenetic protein, allowing the formation of neural cells[14]. Gerrard and colleagues[3] reported that human embryonic stem cells could be induced to differentiate into neural cells under monolayer culture conditions using Noggin to block the bone morphogenetic protein pathway. Increasing evidence confirmed Noggin could act as an antagonist of bone morphogenetic protein, by preventing bone morphogenetic protein from binding homologous receptors on ectoderm cells. It was reported that 500–700 μg/L Noggin is required during the directed induction of neural cells, leading to a high consumption of this expensive factor.

Basic fibroblast growth factor is a polypeptide cell growth factor that exerts strong effects on cell growth and function during neural differentiation in the late stages of development, and is one of the most essential cell factors required for maintaining the self-renewing property of neural stem cells[14-15]. Shen *et al* [16] induced neural cells more efficiently by adding 10 ng/mL basic fibroblast growth factor into DMEM/F12 + B27 medium lacking serum. Other research indicated that basic fibroblast growth factor is an important factor required for the proliferation of neural stem cells[17], and it is commonly used as an exogenous growth factor in neural differentiation. Basic fibroblast growth factor exerts multiple regulatory functions during neurodevelopmental processes, including inhibition of bone morphogenetic protein signaling during the early stages to promote nerve tissue formation.

This study aimed to determine the influence of different factors on the efficiency of neural differentiation from human embryonic stem cells H14, which may pave the way for further optimization of neural induction efficiency *in vitro*. The addition of 20 μg/L basic fibroblast growth factor or 100 μg/L Noggin to serum-free chemically-defined medium promoted the direct differentiation of human embryonic stem cells H14 into neural precursors. This method did not require embryoid bodies or co-culture with feeder layers; therefore, it is convenient as the culture conditions can easily be controlled and changes in cell morphology can be observed by microscopy during the induction period. As the induction time increased, the projections formed by the induced cells gradually became thinner and longer. Moreover, the induced cells showed typical morphological changes associated with neural epithelial cells, including gradual formation of neural rosette structures and weaving of some nerve cells into a network. Significantly greater numbers of neural rosettes formed in the Noggin-induced group, and neural rosettes in the Noggin-induced group were larger than the basic fibroblast growth factor-induced group. In the defined induction environment, different efficiencies of neural induction can be mainly attributed to the specificity of basic fibroblast growth factor or Noggin. Nestin protein was highly expressed in neural rosette structures; therefore, we concluded that the cells in

neural rosettes were neural precursors. Neurofilament expression in the induced cells suggested the generation of functional neural cells; however, these cells may have different functions and features compared to normal adult neural precursors. Further studies are required to determine whether these *in vitro* induced neural precursors have a different plasticity or vitality to normal adult neural precursors. This study demonstrates that Noggin has greater neural differentiation efficiency than basic fibroblast growth factor, leading to a larger quantity of neural precursors in a short period of time.

Although our study confirms that fibroblast growth factor and Wnt signaling pathways participate in directed induction[16], the specific effects of these pathways on the production of primary neural cells or late proliferation of neural cells remain unclear. Moreover, the mechanisms by which Noggin and basic fibroblast growth factor promote human embryonic stem cells differentiation are unknown. We speculate that Noggin and basic fibroblast growth factor may induce human embryonic stem cells to differentiate into neural precursors *via* influencing gene expression.

The primary demand of cell biology and transplantation therapy is to obtain sufficient numbers of neural precursors with uniform traits[18]. In this study, we obtained a large number of neural precursors with a uniform morphology through Noggin-induced neural differentiation of human embryonic stem cells H14 and further cell proliferation, and proved that the cells had the characteristics of neural stem cells. Comparing the different effects of Noggin and basic fibroblast growth factor on human embryonic stem cells H14 could provide a basis for interpreting the molecular mechanisms of *in vitro* directed neural differentiation. Morphological changes in the cells were observed by reverse transcription-PCR and cell immunofluorescence techniques were used to identify neural precursors at the molecular and protein level. Neuroepithelial cells gradually emerged in the Noggin-induced group from day 7, with a high rate of neural rosette structure formation, which had a perfect shape and size, and some neuron-like cells could be observed. The basic fibroblast growth factor-induced group gradually formed neuroepithelial cells from day 10, with a relatively low rate of neural rosette structure formation, leading to small neural rosette structures. These results suggest that Noggin can efficiently induce human embryonic stem cells H14 differentiation into neural precursors. We improved the production of neural precursors from human embryonic stem cells H14 to enable the potential application of these cells in the clinic. Immune rejection and ethical issues could be resolved by somatic cell nuclear transfer, indicating that cell therapy may be realized in the near future.

MATERIALS AND METHODS

Design

A contrast observation of cell biology.

Time and setting

This experiment was performed at the Department of Laboratory Animal Sciences, Shanghai Jiao Tong University School of Medicine, China from April 2010 to November 2011.

Materials

Human embryonic stem cell line H14 was established at the Department of Laboratory Animal Sciences, Shanghai Jiao Tong University School of Medicine, China.

Methods

Culture of human embryonic stem cells

Human embryonic stem cells were cultured according to Amit's methods[19] with a minor modification. In brief, human embryonic stem cells were cultured at 37°C in humidified air with 5% CO_2 in medium composed of Knockout DMEM (Gibco-BRL, Grand Island, NY, USA) containing 20% serum replacement (Gibco-BRL), 2 mmol/L L-glutamine (Hyclone, Logan, UT, USA), 1% MEM non-essential amino acids solution (Gibco-BRL), 0.1 mmol/L β-mercaptoethanol (Sigma, St. Louis, MO, USA), 4 ng/mL basic fibroblast growth factor (Sigma), 50 U/mL penicillin (Gibco-BRL) and 50 mg/mL streptomycin (Gibco-BRL). The neural-inducing basic medium comprised 48% DMEM-F12 (Gibco-BRL), 1% B27 supplement (Gibco-BRL), 49% Neurobasal medium (Gibco-BRL), 1% N2 supplement (Gibco-BRL), 2 mmol/L L-glutamine, 1% MEM non-essential amino acids, 50 U/mL penicillin and 50 mg/mL streptomycin.

Neural induction of human embryonic stem cells

Human embryonic stem cells H14 stored in liquid nitrogen were thawed, human embryonic stem cell media was added and the cells were transferred onto homemade mitotically-inactivated mouse embryonic fibroblasts[20], cultured at 37°C in humidified air with 5% CO_2; the media were changed daily. The cells were expanded every 4–5 days with 4 ng/mL collagenase. Cells from passages 12 to 14 were used in this study and were classified into four groups: based-induced, Noggin-induced and basic fibroblast growth factor-induced (cells

were attached to matrigel-treated Petri dishes), and negative control group (cells were grown on mouse embryonic fibroblasts and maintained in human embryonic stem cells medium). The based-induced group was cultured in N2-B27 medium without growth factors, which was changed every other day. Either 100 μg/L Noggin (Merck, Darmstadt, Germany) or 20 μg/L basic fibroblast growth factor (Merck) was added to the basal medium for Noggin-induced and basic fibroblast growth factor- induced media, respectively. Cell morphology was continuously observed for 3 weeks under FV500 inverted phase contrast microscope (Olympus, Osaka, Japan).

Immunocytochemical analysis of neural cell marker expression in induced cells

Two weeks after induction and the removal of culture medium, the induced cells were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature, washed three times with PBS, treated with 0.05% Triton X-100 for 15 minutes at room temperature, washed three times with PBS, blocked with 3% bovine serum albumin/PBS for 30 minutes at room temperature, and incubated with mouse anti-human nestin monoclonal antibody (1:200; Chemicon, Temecula, CA, USA) and rabbit anti-anti-sox1 (1:10; Chemicon) or β-III tubulin (1:500; Chemicon) polyclonal antibodies. All antibodies were diluted in PBS and incubated overnight at 4°C. The next day cells were washed three times with PBS, incubated with Cy3-conjugated goat anti-mouse or rabbit IgG (1:100; Jackson Immunoresearch, West Grove, PA, USA) at room temperature for 1 hour, washed once with PBS, nuclei counterstained with 4',6-diamidino-2-phenylindole and fluorescent signals visualized and captured using a confocal microscope (FV500; Olympus). The neural precursor differentiation efficiency was calculated as the mean ratio of Nestin positive cells to 4′,6-diamidino-2-phenylindole-stained nuclei in ten randomly selected fields (200-fold) in three independent experiments. The expression of Nestin, Sox-1, β-III Tubulin in neural rosette cells were observed under a confocal microscope, and the number of structures formed was counted in ten fields of view in each group.

Reverse transcription-PCR detection of gene expression of neural cell markers in neural precursors

Two weeks after induction, reverse transcription-PCR analysis was performed using GAPDH as a housekeeping gene. Nestin and Sox-1 are specific markers of neural precursors[4]. The neural rosettes in the Noggin-indu- ced and basic fibroblast growth factor-induced groups were digested into single cells, and gene expression was quantified using reverse transcription-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen, Grand Island, NY, USA) and RNase-free DNase (Promega, Madison, WI, USA), then cDNA was synthesized from 2 μg RNA using random primers and M-MLV reverse transcriptase (Promega) according to the manufacturer's protocol.

The primers and annealing temperatures are described as follows:

Gene	Primer sequence (5′–3′)	Annealing temperature (°C)	Cycle	Product size (bp)
Sox1	Forward: CAA TGC GGG GAG GAG AAG TC Reverse: CTC TGG ACC AAA CTG TGG CG	55	28	464
Nestin	Forward: GAG GAC CAG AGT ATT GTG AGA C Reverse: CAC AGT GGT GCT TGA GTT TC	58	28	368
Neurofilament	Forward: ACC CGA CTC AGT TCA CCA Reverse: TTC TTC ACC TTC ACC TCC TTC	55	26	357
GAPDH	Forward: AGC CAC ATC GCT CAG ACA CC Reverse: GTA CTC AGC GCC AGC ATC G	55	20	303

PCR was performed by denaturation at 94°C for 2 minutes; a suitable number of cycles of 30-second denaturation, 30-second annealing, 30-second extension at 72°C; followed by a final 5 minute extension at 94°C and holding at 4°C. The amplification products were electrophoresed on 2% agarose gel and were stained with ethidium bromide (Sigma).

Statistical analysis

Data were expressed as mean ± SD, and were analyzed using SAS 8.1 software (SAS Institute Inc. Cary, NC, USA). The two-sample *t*-test was used to analyze the data. *P* values < 0.05 were considered statistically significant.

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