

## Forskolin cooperating with growth factor on generation of dopaminergic neurons from human fetal mesencephalic neural progenitor cells

Xuan Wang<sup>a</sup>, Xiaoxia Li<sup>b</sup>, Kun Wang<sup>a</sup>, Huifang Zhou<sup>a</sup>, Bing Xue<sup>a</sup>,  
Linsong Li<sup>b</sup>, Xiaomin Wang<sup>a,c,\*</sup>

<sup>a</sup>Neuroscience Research Institute, Peking University, Beijing 100083, PR China

<sup>b</sup>Stem Cell Research Center, Peking University, Beijing 100083, PR China

<sup>c</sup>Department of Physiology, Capital University of Medical Sciences, Beijing 100054, PR China

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### Abstract

Forskolin was tested for its co-activating ability to enhance the function of fibroblast growth factor (FGF) 8 on dopaminergic (DAergic) differentiation from human fetal mesencephalic neural progenitor cells (NPCs). When NPCs were treated with FGF8 alone, the DAergic phenotype was expressed lightly. The addition of 10  $\mu$ M forskolin increased the number of DAergic neurons, cooperating with 50 ng/ml FGF8. These cells produced neurotransmitter DA, which was measured by high-performance liquid chromatography. Reverse transcriptase-polymerase chain reaction analysis demonstrated that differentiated cells expressed DAergic development-related genes tyrosine hydroxylase (TH), nuclear receptor-related factor 1 (Nurr1) and D2 receptor (D2R), indicating that matured DAergic neurons could be obtained under these present conditions. The results suggest that forskolin plus FGF8 may contribute to more efficient production of DAergic neurons from human-derived NPCs for therapy of neurodegenerative diseases.

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Neural progenitor cells (NPCs) have been demonstrated to be a population with self-renewal and multi-differentiation properties [12]. Under certain condition, these cells can be controlled to differentiate committedly into specific neuronal phenotypes, for example, dopaminergic (DAergic) neurons, which are the major cell type damaged in Parkinson's disease (PD). Several groups have successfully isolated NPCs from the fetal rodent [10] and human mesencephalons [18], where DAergic neurons are formed during normal developmental procedures. However, these cells rarely converted into the DAergic phenotype spontaneously, suggesting that environmental factors or epigenetic stimulations are needed to complete the differentiation process. If the factors responsible for the DAergic differentiation could be identified, it might be possible to regulate the committed differentiation so that NPCs

enriched in a DAergic phenotype could be generated for neural transplantation therapy of PD. To date, many research results have indicated that, contrary to primary non-converted NPCs, pre-differentiation in vitro is a key step in improving the disordered behaviors of parkinsonian animal models after transplantation [3]. Although the generation of specific desired neuronal phenotype in vitro from dividing progenitor cells seems to be quite difficult, some cytokines have been proven to significantly increase the differentiation capacity of progenitor cells into DAergic neurons. These cytokines, such as fibroblast growth factors (FGFs) [17], sonic hedgehog (Shh) [8], interleukin-1 (IL-1) [3,10,16] and bone morphogenetic proteins (BMPs) [1], were shown to play important roles in up-regulating the expression of tyrosine hydroxylase (TH), the first and rate-limiting enzyme in the procedure of DA synthesis. However, a method for large-scale production of DAergic neurons from human fetal mesencephalic progenitor cells remains to be found.

One critical factor in the growth and polarity of the developing midbrain is fibroblast growth factor 8 (FGF8),

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\* Corresponding author. Tel.: +86-10-8280-2411; fax: +86-10-8280-1111.

E-mail address: xmwang@bjmu.edu.cn (X. Wang), xmwang@cpums.edu.cn (X. Wang).

which was found to significantly promote the differentiation of embryonic stem cells (ES cells) into DAergic neurons [8]. Some studies have also shown that FGF8 is involved in the determination of a DAergic neuronal cell fate in vivo and in neuronal protection against oxidative stress in cultured hippocampal neurons [11]. However, for human fetal mesencephalon-derived progenitor cells, it is not known whether FGF8 can increase DAergic differentiation and play its neurotrophic role. Recently, several co-activators that influenced the DAergic differentiation have been reported. These co-activators included DA, cyclic AMP (cAMP), phorbol 12-myristate 13-acetate (TPA), isobutylmethylxanthine (IBMX) and forskolin [4,5]. In rodent investigations, when one of the co-activators was added into differentiation medium, it could magnify the induction effect of growth factors on TH expression. In our present research, we examined the maximal effect of FGF8 alone on the production of TH-immunoreactive (TH-IR) neurons. Furthermore, FGF8 was administered in combination with forskolin to test possible additive effects on the DAergic differentiation of human-derived mesencephalic NPCs. These data suggest that epigenetic cues may be crucially effective in promoting the differentiation of a DAergic phenotype in human-derived NPCs needed for clinical transplantation therapy for PD.

Mesencephalic NPCs were obtained from a natural aborted human fetus of 9 weeks of gestation, and prepared as described previously [20]. Permission to use human embryonic tissue was granted by the ethical committee of Peking University and with the patient's consent. Briefly, the ventral mesencephalon was dissected, washed with cold D-Hank's solution and minced thoroughly. Tissue fragments were mechanically separated into single cells and were cultured in serum-free neurosphere-forming medium, Dulbecco's modified Eagle medium (DMEM)/F12 (1:1), containing a supplement of B27 (1:50, Gibco Life Technologies, Rockville, MD), 20 ng/ml epidermal growth factor (EGF, Gibco), 20 ng/ml human recombinant basic fibroblast growth factor (bFGF, Gibco) and 10 ng/ml leukemia inhibitory factor (LIF, Chemicon, Temecula, CA). After floating neurospheres were formed in several days in vitro (DIV) cultivation, dividing cells were mechanically dissociated and reseeded onto fresh medium. Neurospheres were passaged every 2 weeks in order to purify the long-term propagating progenitor cells. After four passages, proliferative neurospheres were gently triturated and transferred onto a poly-L-lysine-coated 48-well plate at a density of  $10^4$ /well in the basic differentiation medium, DMEM/F12 (1:1) containing 10% fetal bovine serum (FBS), after EGF, bFGF and LIF withdrawal. Cultures were incubated with different doses of recombinant human FGF8 (10, 50, 100, 200 ng/ml; Gibco) in order to test the effect of FGF8 alone. Then 10  $\mu$ M forskolin (Sigma, St. Louis, MO) was added into the differentiation media alone and with the different doses of FGF8 indicated in the above experiment to test possible additive effects of the co-

activator on neurotrophic factor. For one experiment, the same batch of neurospheres was used and non-treated cells were set as the negative control group. All cytokines were added to the medium for an incubation period of 7 DIV. Induced cultures were fixed with 4% paraformaldehyde, and processed for TH immunostaining (mouse anti-TH monoclonal antibody, diluted 1:500, Sigma). TH-IR cells were counted and the result was expressed as a percentage of TH-IR cells found in paired control group cultures.

To further characterize the function of differentiated TH-IR cells, the capacity to synthesize and release DA was assessed by reverse-phase high-performance liquid chromatography (HPLC). The differentiated medium containing forskolin and FGF8, and incubating with the cells for 7 DIV, proved to be the most effective conditions for culturing NPCs. DA release was stimulated in HBSS containing 56 mM KCl for 15 min incubation as described previously [17]. In brief, at the appropriate medium change, a sample of the culture medium was taken. Protein in the samples was precipitated with 0.4 M HCl, and then the sample was centrifuged at  $2500 \times g$  for 10 min at 4 °C followed by HPLC analysis. In order to evaluate mRNAs expression of specific genes, TH, nuclear receptor-related factor 1 (Nurr1) and D2 receptor (D2R), which are involved in DAergic neuron development and function, the same factors as those used for HPLC experiment were added to the cultures at 7 DIV. Then a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. The following primers were used to amplify target cDNA: TH (206 bp), 5'-TTCGCGCAGTTCTCGCA-3' and 5'-AAGGCCCGAATCTCAGGCT-3'; Nurr1 (245 bp), 5'-ACTCCAACCCGGCTATGACC-3' and 5'-GGACCTGTATGCTAATCGAAGGA-3'; D2R (201 bp), 5'-CAAGACCATGAGCCGTAGGAAG-3' and 5'-TTCACGGCGCTGTTGACATAAG-3'; GAPDH (452 bp), 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. After amplification, the products were separated on an agarose gel in the presence of ethidium bromide and visualized under UV light. Statistical differences between groups (mean  $\pm$  SEM) were tested using one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test ( $P < 0.05$ ). The statistical difference between the two groups (mean  $\pm$  SEM) was tested by *t*-test.

Human fetal mesencephalic NPCs formed proliferating neurospheres after 2 weeks cultivation in vitro. The NPCs were passaged by gently triturating the neurospheres, and after four passages of expansion, the total cell number increased from  $45000 \pm 305$  to  $1354000 \pm 21000$  ( $n = 5$ ). When removing EGF, bFGF and LIF from the culture media, NPCs began to differentiate spontaneously under the induction condition containing 10% FBS in the control group. However, in this control group, only a few TH-IR cells were observed (see Fig. 2A). FGF8 alone had a positive induction effect on DAergic neurons differentiation compared to the control cultures. The numbers of TH-IR

cells apparently increased when the cells derived from neurospheres were incubated with 50, 100 and 200 ng/ml FGF8. The percentage of TH-IR cells was 169.5% ( $P < 0.001$ ) in 50 ng/ml FGF8, 141.5% ( $P < 0.05$ ) in 100 ng/ml FGF8 and 142.5% ( $P < 0.05$ ) in 200 ng/ml FGF8, respectively, relative to the control cultures (Fig. 1A,  $n = 5$ ). Forskolin alone (10  $\mu$ M) did not influence the number of TH-IR cells, which had no difference from that of control cultures. When the neurospheres were treated with a combination of 10  $\mu$ M forskolin and different concentrations of FGF8, forskolin induced a significant increase in the number of TH-IR cells per neurosphere, particularly when cooperating with 50 ng/ml FGF8 (272.1% of the control cultures,  $P < 0.001$ ). The same effects were also obtained at doses of 100 ng/ml (218.1%,  $P < 0.001$ ) and 200 ng/ml (218.2%,  $P < 0.001$ ). Nevertheless, the difference in the number of TH-IR cells was apparent between the cultures induced by the forskolin combined with 50 ng/ml FGF8 and with 100 ng/ml ( $P < 0.01$ ) or 200 ng/ml ( $P < 0.01$ ) FGF8 (Fig. 1B,  $n = 5$ ). Most of the differentiated TH-IR cells appeared as mature neurons with long, branching processes, and there were only a few with an immature appearance (Fig. 2C). All the TH-IR cells (Fig. 2D) co-expressed  $\beta$ III-Tubulin (Fig. 2E,F), which proved their neuronal trait.

We selected the most effective dose, i.e. 10  $\mu$ M forskolin

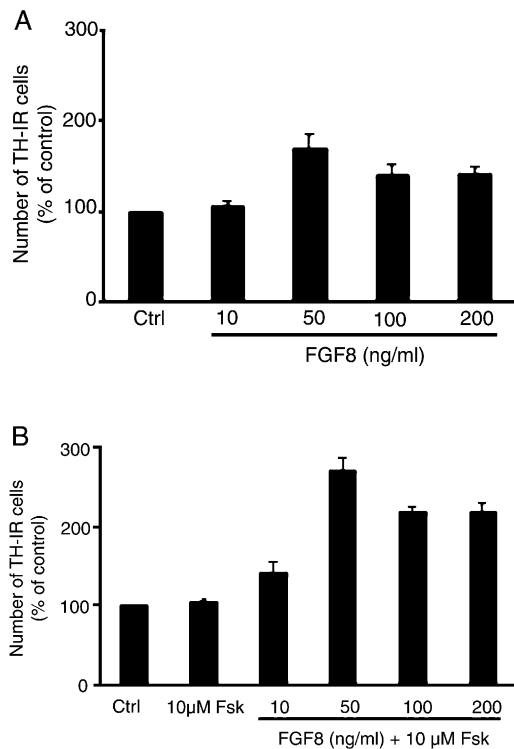


Fig. 1. Effects of different doses of FGF8 alone (A) and combined with 10  $\mu$ M forskolin (B) on the number of TH-IR cells differentiated from neurospheres of human fetal mesencephalic NPCs after 7 DIV incubation. The data are expressed as percentages of the number of TH-IR cells obtained in the respective control cultures (100%) and values are presented as mean  $\pm$  SEM. Ctrl, control; Fsk, forskolin.

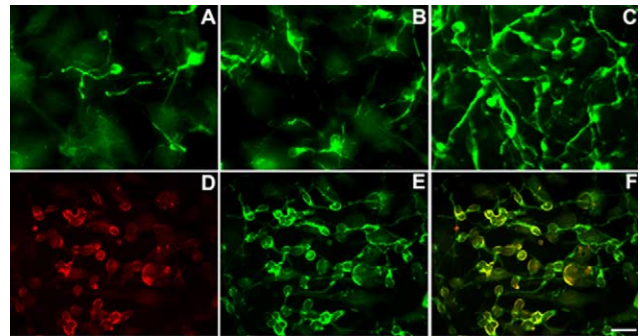


Fig. 2. FGF8 at the dose of 50 ng/ml increased the TH expression (B) contrary to the control (A). The addition of 10  $\mu$ M forskolin increased the number of TH-IR cells (C), cooperating with 50 ng/ml FGF8. Under this condition, all the TH-IR cells (red in D) expressed  $\beta$ III-Tubulin (green in E). Double-positive signals are presented in (F) (yellow). Scale bar, 20  $\mu$ m.

and 50 ng/ml FGF8, to further test the DA production by these neurons. The result by HPLC ( $n = 5$ ) showed that after treatment with cytokines, the level of DA in the supernatants increased significantly ( $1.47 \pm 0.20$  ng/ml), compared with the control cultures ( $0.05 \pm 0.02$  ng/ml). The evoked release was about 28-fold increased ( $P < 0.01$ ). These results demonstrated that a combination of forskolin and FGF8 increased the yield of functional DAergic neurons. In order to investigate the molecular mechanisms of forskolin and FGF8 on the induction of DAergic differentiation, semi-quantitative RT-PCR was used to assay expressions of candidate genes TH, Nurr1 and D2R, which were critical for DAergic neuron differentiation during the developmental procedure (Fig. 3). While a low, but detectable, level of TH mRNA was present, expression of Nurr1 and D2R mRNAs was not detectable in the control group. FGF8 alone up-regulated the expression of TH and Nurr1 mRNAs. After incubation with 10  $\mu$ M forskolin and 50 ng/ml FGF8, differentiated cells expressed higher levels of TH and Nurr1, and began to express D2R.

Production of DAergic neurons from rodent NPCs has been discussed fully and accurately, whereas the method and

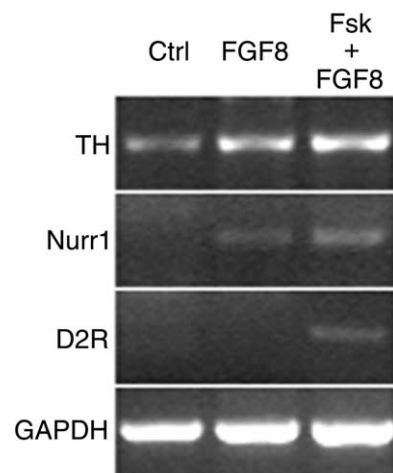


Fig. 3. Forskolin cooperating with FGF8 increased the mRNAs of these candidate genes relative to control or FGF8 administrated alone.

mechanisms of inducing human-derived NPCs to differentiate into DAergic neurons in vitro were reported only in one or two study groups [14,15]. For clinical applications, human-derived NPCs possess greater advantages than those from animals. Therefore, we need to know more about multiple variables that may increase the generation of DAergic neurons with DAergic phenotype and normal function from human mesencephalic NPCs. Our present study showed that FGF8, which was the presumptive differentiator of DAergic neurons in vivo, could increase the number of TH-IR cells in human fetal mesencephalic NPCs grown in vitro, although this induction effect was relatively weaker than expected. The level of TH induction by FGF8 could be enhanced by the addition of one of the competent co-activators, forskolin, which suggests that the additive effect of co-activators on the growth factors occurs in the NPC system of the present studies. Furthermore, a more important facet we need to know is whether the differentiated TH-IR cells are the functional DAergic neurons, whose apparent property is to be able to produce the neurotransmitter DA as well as the phenotypic characteristics. Increasingly measurable amounts of DA detected by HPLC in the culture media suggested that controlled differentiation of NPCs by the combination of FGF8 and forskolin led to the induction of catalytically active TH.

Furthermore, our research is amongst the first studies regarding the induction mechanism of growth factor FGF8 and its co-activator forskolin on DAergic differentiation from human fetal mesencephalic NPCs. The induced expression and/or increased expression of some certain genes may play an important role during the differentiation procedure. The RT-PCR result showed that when treated with the synergist of forskolin and FGF8, cells expressed the higher levels of TH, Nurr1 and D2R mRNAs compared to control and FGF8 alone treatments in the present study. The TH gene contains several *cis*-acting elements upstream of the start site, such as the cAMP response element (CRE) [19] and the activator protein 1 (AP-1) [6], which are important for TH gene expression [2]. The possible induction mechanism of FGF8 is that after binding to its receptor it activates an intracellular signaling pathway leading to an increase in Fos/Jun binding to the AP1 regulatory site on the TH gene [6]. However, the results from the present study indicated that FGF8 alone might be less effective than the combination with forskolin, and thus cannot adequately derepress the gene as is required for TH expression during the differentiation process. This induction of TH gene expression by FGF8 could be amplified by cooperating with its co-activator forskolin, which can up-regulate the level of cAMP. Increases in levels of cAMP activate cAMP-dependent protein kinase (PKA), phosphorylate several target proteins that bind specifically to CREs and exert critical control of transcription of the TH gene [7, 9]. Therefore, except for the effect of FGF8 on the AP-1 site, the rise of TH protein expression was also probably due to an increase in cAMP levels induced by forskolin and so up-regulating TH mRNA synthesis through the CRE on the TH

gene. In addition to TH, the expressions of Nurr1 and D2R mRNAs were up-regulated by the FGF8 and forskolin used in concert. These genes are involved in DAergic neuron development and function, and their expressions suggest that the matured differentiated DAergic neurons can be obtained in our present inducing system [13].

The results presented here show that the growth factor FGF8 alone can induce the DAergic neuron differentiation from human-derived NPCs. A significantly higher degree of induction of the DAergic phenotype is achieved upon addition of the adenylyl cyclase activator, forskolin, into the differentiation culture in the presence of FGF8. The percentage of DAergic neurons in the present conditions indeed needs to be modulated to further increase the DAergic neuron population, however, our results demonstrated that forskolin plays the co-activator role on growth factors and these extrinsic cues may be crucially effective in promoting the differentiation of a DAergic phenotype in human-derived NPCs. Ultimately, clinically available differentiated human NPCs as transplant cells will provide an effective therapeutic treatment of PD.

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