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Activation of TGFβ1 signaling enhances early dopaminergic differentiation in unrestricted somatic stem cells

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HIGHLIGHTS

- We investigated the role of TGFβ signaling in neural differentiation of USSCs.
- Components of TGFβ signaling were present and functional in undifferentiated USSCs.
- Activation of TGFβ signaling in USSCs enhances expression of neural markers.
- TGFβ signaling is involved in early dopaminergic differentiation of USSCs.

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ABSTRACT

So far there is increasing evidence for the involvement of transforming growth factor beta TGFβ (transforming growth factor) in differentiation and maintenance of midbrain dopaminergic neurons. Considering that USSCs (unrestricted somatic stem cells) have the potentials to differentiate into neuron-like cells and even dopaminergic neurons and that no evidence available on the role of TGFβ signaling in dopaminergic differentiation of these cells, we investigated the presence of TGFβ signaling components in USSCs and their involvement on USSCs differentiation into early dopaminergic neurons. Our results showed that components of TGFβ signaling were present and functional in undifferentiated USSCs, after which the neurally induced USSCs treated with TGFβ1 for 3 days resulted in increased expression of β-tubulin III (a general neuronal marker) and Nurr-1 (an early dopaminergic marker) at both mRNA and protein levels. Consistently, TGFβ inhibition in culture medium by using SB431542 in the presence or absence of TGFβ1, significantly decreased the expression of both neural markers. We therefore suggest that activation of TGFβ signaling-pathway in neurally induced USSCs enhances neural differentiation with an early dopaminergic phenotype which points at the positive role of the TGFβ signaling pathway toward dopaminergic fate.

1. Introduction

The loss of dopaminergic neurons located in substantia nigra is considered as the main cause of Parkinson's disease. Since cell replacement-based therapy is considered as the best therapeutic strategy for this disease, it is important to understand the mechanism underlying the dopaminergic neuron differentiation and the signaling pathways that control their differentiation.

Increasing evidence indicates that transforming growth factor beta (TGFβ) is involved in differentiation and maintenance of midbrain dopaminergic neurons [1]. Members of the TGFβ family bind to the complex receptors including type I and II receptor serine/threonine kinase. Type II receptor activates type I which then activate Smads molecules to transduce the signal. While TGFβ, nodal and activin members of family activates Smad 2 and 3, bone morphogenetic protein (BMP[1]) activates Smads 1, 5 and 8. These

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[1] Bone morphogenetic protein.

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activated Smads bind with Smad 4 to make active transcription factor [2].

Large body of evidence indicates that inhibition of TGFβ/activin/nodal signaling is required for induction of human embryonic stem cells (hESC[2]) into a neural fate [3–9]. However, there are also reports showing that TGFβ signaling enhances neuronal maturation and dopaminergic differentiation of neural progenitor cells [10–12].

Newly introduced stem cell called unrestricted somatic stem cells (USSCs) can be isolated from human cord blood at birth, specified as CD45 and HLA[3] class II-negative cell [13]. Non-invasive collection and low risk of forming teratomas in an animal model have made USSCs as attractive sources of stem cells for cell therapy. These cells have a high *in vitro* proliferative capacity and the ability to differentiate into osteo-, chondro-, hematopoietic and neural cells *in vitro* [14,15].

It has been shown that USSCs have the potential to differentiate into neuron-like cells and even dopaminergic neurons [16–18] when cultured in the presence of neural inducing medium. However, there is almost no information on the signaling events regulating the self-renewal and differentiation of USSCs. We previously reported the only evidence of the presence of functional components of Wnt/β-catenin signaling in these cells, suggesting a role for this pathway in neural differentiation [16].

The aim of the present study is to examine presence of TGFβ signaling components in USSCs and their involvement in differentiation of USSCs into the early dopaminergic neurons. In this project, TGFβ1 recombinant protein and its inhibitor (SB431542) were used to activate and inhibit this signaling pathway, respectively. Our results showed that TGFβ signaling components were present in USSCs and induction of this pathway enhanced the early dopaminergic differentiation of neurally induced USSCs.

2. Materials and methods

2.1. Chemicals

Retinoic acid, IBMX[4] (3-isobutyl-1-methylxanthine) and bFGF[5] (basic fibroblast growth factor) were purchased from Sigma (UK). Human recombinant TGFβ1 and SB431542 were purchased from Peprotech (UK) and TOCRIS (UK), respectively. Western blot detection kit was purchased from Roche Applied Science (Germany); nitrocellulose membrane from Whattman; Phospho Smad2, Smad2/3 antibodies from Cell Signaling Technology (USA); β-tubulin III antibody from Sigma (UK); Nurr1 antibody from Santa Cruz (USA), FITC conjugated secondary antibody from Razi biotech (Iran); DMEM, Fetal Bovine Serum (FBS[6]), penicillin–streptomycin, trypsin and EDTA from Gibco (UK); and Biomax film from Kodak (UK). All other chemicals were purchased from Merck (Germany).

2.2. USSC isolation and culture

Isolation, culture and characterization of human USSCs were performed as described by Kögler et al. [13]. Umbilical cord vein of the informed consented mothers was considered as a source of cord blood from which the mononuclear cell fraction was separated by density centrifugation of a Ficoll gradient. USSCs were successfully extracted at a rate of 40% from cord blood samples, expanded in low glucose DMEM supplemented with 20% FBS (control medium) in a humidified incubator with 5% CO_2 at 37 °C. Following 80% confluency, the cells were passaged and replated after detachment with 0.25% Trypsin-EDTA.

Table 1
Primers used for real-time PCR.

Gene	Primer sequences (sense, top; antisense, bottom)	Amplicon length
Nurr-1	5-GCC CAT GTC GAC TCC AAC-3 5-ACT CAT TTG ATA GTC AGG GTT CG-3	78
β-tubulin III	5-GCA AAC TAC GTG GGC GAC T-3 5-CGA GGC ACG TAC TTG TGA GA-3	85
β2M	5-CTA TCC AGG GTA CTC CAA AG-3 5-GAC AAG TCT GAA TGC TCC AC-3	105

2.3. Neural differentiation

Human USSCs were induced to differentiate into neural cells in neural induction medium (further referred as neural medium) for 3 days. USSCs were cultured in 25 cm^2 flasks at a density of 4000 cells/cm^2, and treated with differentiation medium [13] containing DMEM supplemented with 7.5% FBS, 1% penicillin/streptomycin, 20 ng/ml bFGF, 0.5 mM IBMX, 50 μM ascorbic acid and 10 μM retinoic acid for up to 10 days. Culture medium was changed every 2–3 days.

2.4. Western blotting

USSCs were washed with ice-cold PBS. Total cell lysate was prepared using protein lysis buffer (Tris 62.5 mM, pH 6.8, DTT 50 mM, NaF 10 mM, SDS 2% (w/v), protease/phosphatase inhibitor cocktail (100×) and stored at 20 °C. Subsequently, 10 μg of each sample was subjected to 10% SDS-PAGE gel electrophoresis. The gels were blotted onto a nitrocellulose membrane and blocked with 3% BSA, 0.03% (v/v) Tween 20 in TBS[7] (tris buffered saline) at room temperature for 1 h. The membrane was probed with Smad2/3 (1:1000) and phospho Smad 2 (1:1000) as primary antibodies. The membranes were subsequently probed with corresponding HRP-conjugated secondary antibodies at a 1:20,000 dilution. Signals were visualized using chemiluminescence on the Biomax film. Densitometry analysis used Scion Image (Version 4.0.2; Scion Corporation, USA).

2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized with Triton X-100 (0.3%) and probed using primary antibodies to β-tubulin III (1:250), Nurr-1(1:200) and FITC-conjugated anti-rabbit secondary antibody (1:60).

2.6. Real-time PCR

Real-time PCR[8] (polymerase chain reaction) was performed in a single-color real-time PCR detection system using a syber green reaction mix (Qiagen) and an equivalent of 40 ng RNA of each sample with primer. Data were normalized to β2M and are presented as fold-change with respect to control-treated samples. The primers and product lengths are listed in Table 1.

[2] Human embryonic stem cells.
[3] Human leukocyte antigen.
[4] 3-Isobutyl-1-methylxanthine.
[5] Basic fibroblast groeth factor.
[6] Fetal bovine serum.
[7] Tris buffered saline.
[8] Polymerase chain reaction.