

# TGF- $\beta$ signaling in neuronal stem cells

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**Abstract.** Transforming growth factor beta (TGF- $\beta$ ) signaling has diverse and complex roles in various biological phenomena such as cell growth, differentiation, embryogenesis and morphogenesis. ES cells provide an essential model for understanding the role of TGF- $\beta$  signaling in lineage specification and differentiation. Recent studies have suggested significant role of TGF- $\beta$  in stem/progenitor cell biology. Here in this review, we focus on the role of the TGF- $\beta$  superfamily in neuronal development.

## 1. Introduction

Neuronal precursor cells that have the capacity for self-renewal and ability to produce daughter cells which are more differentiated along one of several (neuronal/glia/oligodendroglial) lineages are regarded as neuronal stem cells (NSC).

For its maintenance and development, the neuronal stem cell depends upon a reciprocal relationship with its environment. The neuronal stem cell niche is its microenvironment, containing those factors that are needed to regulate stem cell expansion *in vivo* [1]. In the brain, neuronal stem cells (NSCs) are thought to be located in discrete anatomical regions including the ventricular and subventricular zone of the lateral ventricle, the subgranular zone of the hippocampal dentate gyrus as well as the olfactory cortex [2,3].

Before development of the stem cell niche, however, stem cells in the gastrula stage of the embryo lay down anterior or posterior ectoderm, mesoderm and endoderm germ cell layers under the influence of several factors. Most importantly, TGF- $\beta$  itself does not appear to be involved at this stage although other members of the TGF- $\beta$  family, such as BMP, Cripto and

Nodal do have strategic roles in the gastrula and early embryonic stages of neuronal development. Using Embryonic Stem (ES) cells derived from the inner cell mass of the blastocyst that precedes gastrulation, or whole embryos carrying single gene mutations, investigations have revealed a key role for BMPs in maintaining ES cell self-renewal. Leukemia Inhibitory Factor (LIF) has been shown to inhibit epidermal differentiation of ES cells and may promote neural differentiation, while simultaneous signaling from BMP induces Id gene mediated inhibition of neural differentiation [4]. Cripto/Nodal, Wnt and FGF signals have important functions in the early embryo and the blastula stage for induction of posterior mesodermal patterning and maintenance/renewal of anterior stem cell population. Loss of Cripto function in *Cripto*<sup>-/-</sup> ES cells also results in enhanced neurogenesis compared to wild type ES cells [5]. However, disruption of Cripto alone is not sufficient to initiate “default neurogenesis” because *Cripto*<sup>-/-</sup> ES cells differentiate into multiple lineages of cell types. Blocking Nodal or *wnt* signaling, in ES cells with *Dkk1* and *LeftyA*, respectively, results in further enhancement of neural differentiation, whereas gain-of-function of Nodal results in inhibition of neuroectoderm development [6]. Furthermore, *wnt* signaling and BMPs as well as extracellular matrix components induce non-neural differentiation of ES cells [7]. Useful markers of germ cell layers that are influenced by BMP, Wnt, FGF and LIF and their signaling intermediates are *brachyury* for the mesoderm [8], *HNf4*

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for the late endoderm, *GATA4* for the early endoderm, *Nestin* and *Tuj1* for the neuroectoderm.

NSCs produce progenitor cells through asymmetric division; one of the two daughter cells resulting from cell division remains a stem cell and the other differentiates to become a progenitor cell that may differentiate further, ultimately becoming a neuron, astrocyte, or oligodendrocyte. In a variation of cell division occurring in NSCs, a daughter progenitor cell undergoes apoptosis while the other survives as a more differentiated cell [9–11]. Each cell division appears to bring the daughter cell or cells closer to the point of restricted or limited potential for further differentiation. Regulation of NSC cell-cycle involves interplay of several signaling pathways including FGF, TGF- $\beta$ , Notch, and Wnt.

The question, how nerve cells are generated from multipotent stem cells, is still debated [12]. However, emerging data suggest that it occurs by “default”, and by exogenous inhibitory factors (either soluble or paracrine). Cell adhesion proteins also play an important role in this process [12,13]. Several investigations provide evidence for an important role for TGF- $\beta$  in this orchestration of signals. For example, *Smad4*<sup>-/-</sup> and *elf*<sup>-/-</sup> mice exhibit a marked increase in cerebellar precursor cells [20] and TGF- $\beta$  mutants show increased neuronal apoptosis [14–16].

This review will focus on TGF- $\beta$  regulation of NSCs, including the dual control of cell-cycle via TGF- $\beta$ /Notch regulation.

## 2. Overview of TGF- $\beta$ signaling

The TGF- $\beta$  superfamily represents nearly 30 growth and differentiation factors that include TGF- $\beta$ s, activin/inhibins, nodal/cripto and bone morphogenetic proteins (BMPs) [17–22]. TGF- $\beta$  signals are transmitted through two types of receptors (type I and type II), both of which are transmembrane serine/threonine (Ser/Thr) kinases [23–25]. Type II receptors, which are thought to be constitutively phosphorylated, are comprised of 5 members (BMPRII, ActRII, ActRIIB, TBR1, AMHR) and show a high affinity for TGF- $\beta$  and Activin proteins. Upon ligand binding of the extracellular domain, type II receptor goes through conformational change and forms a complex with the type I receptor which ultimately facilitates phosphorylation of the type I receptor. The activated type I receptor, in turn, phosphorylates T $\beta$ RI-associated Smads, Smad2 and Smad3, which form a heterodimeric complex with

the co-mediator Smad, Smad4, and translocates into the nucleus to activate or suppress target gene expression [26,27]. Activity of Smad proteins is modulated by a multitude of other proteins that include adaptors such as Embryonic Liver Fodrin (ELF), Filamin or Smad anchor for receptor activation (SARA), and E3 ligases such as SMURFs or PRAJA [20,21, 28–30]. By inducing p53 N-terminal phosphorylation, RTK/Ras/MAPK (mitogen-activated protein kinase) has been shown to facilitate interaction of p53 with the TGF- $\beta$ -activated Smads. As described in study of markers of embryonic mesoderm in *Xenopus* embryos [31], this cooperation between a mitogenic pathway and a cytostatic pathway illustrates one of the many mechanisms by which apparently antagonistic extracellular signals promote TGF- $\beta$  cytostasis in human cells. These data indicate a mechanism to allow extracellular cues to specify the TGF- $\beta$  gene-expression program. It has long been understood that BMPs are important in neuronal development, however, few studies, prior to the studies of *elf* mutant mice, have implicated TGF- $\beta$ . Although many experiments lead to the conclusion that TGF- $\beta$  has relatively few active roles at early stages of development of the brain, these might reflect absence of TGF- $\beta$  receptor expression in the developing embryonic brain.

## 3. ELF regulation of NSCs

An accumulation of data over the past decade has shown that TGF- $\beta$  signaling plays a crucial role in morphogenesis including lineage specification during brain development [12,32]. It is hypothesized that TGF- $\beta$  facilitates lineage commitment in precursor cells supported by the data that lineage-uncommitted stem cell/precursor cells (LUSC), without TGF- $\beta$ , maintain their phenotype. We found that loss of TGF- $\beta$  signaling in *elf*<sup>-/-</sup> mice produced neuronal precursor cells lacking expression of lineage-specific markers such as  $\beta$ -tubulin and nestin. ELF is a 200 kD  $\beta$ -spectrin protein that, along with Smad3 and Smad4, transmits TGF- $\beta$  signaling internally. Importantly, this protein complex may target the transcription of CDK inhibitors such as p21, p15, and p27 thus exerting control upon G1/S cell-cycle progression. ELF was originally identified as a key protein involved in endodermal stem/progenitor cells committed to foregut lineage. It also has been found to be a marker for neuronal precursor cells in the developing mammalian brain [33]. ELF appears to have a novel role because, in the cerebellum, unlike G-

spectrins which are expressed in axons and cell bodies only, ELF is expressed also in the dendrites of Stellate cell precursors and Purkinje cell precursors [34, 35]. Additionally, one variant of ELF (ELF3), a novel  $\beta$ -spectrin, is known to be involved in axonal guidance and cell polarization [33,36–38].

Embryonic lethality has been a common phenotype for mutation of many genes associated with stem cell regulation that include TGF- $\beta$  pathway members such as *elf* [39]. Although the mutation produces embryonic lethality, study of *elf*<sup>-/-</sup> fetal brain demonstrates the impact of loss of ELF on several facets of cell-cycle regulation. Indeed, embryonic *elf*<sup>-/-</sup> brains were found to be hyper-proliferative (particularly marginal cell hyperplasia) with CDK4 induced hyper-phosphorylation of Rb, p130 and p107 and concomitant loss of p21 induced cell cycle control. Furthermore, expression of nestin, the intermediate filament associated protein found in neural stem cells, was lost in these knockout mice [52].

#### 4. Notch signaling: TGF- $\beta$ crosstalk in NSC regulation

Notch signaling has been demonstrated to promote NSC survival [40,41] making the apparently antagonistic roles of Notch/TGF- $\beta$  signaling in the maintenance of NSCs interesting for investigators. The canonical Notch signaling pathway, like TGF- $\beta$ , has been demonstrated to be highly complex and cell type specific and recent evidence suggests that it is in fact bi-directionally regulated, though unlike activation with a cytokine (TGF- $\beta$ ), canonical Notch activation requires neighboring cell contact between Notch ligand and receptor. To date, there are four mammalian Notch receptors, which are single transmembrane receptors (Notch1-4) that interact with five different ligands (Jagged1-2, Deltalike1-2, 4) [42].

Notch activation is transmitted internally through a series of two proteolytic cleavages, first by a protease from the ADAM metalloprotease-disintegrins family and subsequently by  $\gamma$ -secretase cleavage [43]. These cleavages in turn release the Notch intracellular domain (ICD) and allow it to translocate into the nucleus and form a transcriptional activation complex to effect target gene activation [44]. One well known Notch target gene in humans is the mammalian analogue to the Hairy enhancer of split [45] gene that was first characterized in *Drosophila*. Investigations by Ishibashi et al. have

shown a role for this Notch target as a transcriptional repressor of neuronal and glial differentiation cues [46].

One potential point of convergence between the two pathways is in the regulation of c-myc, a target gene of both pathways. In epithelial cells, Notch disrupts the CDK inhibitor p15<sup>INK4B</sup> which is then unable to inhibit c-myc [47].

Of note is how the two pathways functionally interact through the regulation of Smad proteins. For example, Delta-like protein intracellular domain (Dll1IC), once activated through cleavage, specifically binds to Smad2, Smad3, and Smad4, and enhances Smad-dependent gene activation in mice [48]. Thus, the adaptor protein ELF, as a substrate of Smad2, Smad3, and Smad4 [49] may provide a pivotal stabilizing role in the proper signaling of Notch/TGF- $\beta$ .

#### 5. Asymmetric and symmetrical cell division

Asymmetric cell division is not the only type of cell-division executed in the NSC, but is used as a facultative process for simultaneous maintenance of stem cell population and differentiation or apoptosis. For the alternative process of stem cell expansion, the neuronal stem cell uses symmetric division. Regulation of NSC cell-cycle and cell-cycle exit for apoptosis or differentiation is a complex process and is subject to the influence of inductive factors that are usually non-cell autonomous but may be cell autonomous. Inductive factors that influence these processes are often the result of bi-directional signaling with adjacent cells and the type of inductive influence changes as embryonic development advances. Examination of the brains of mutant mice lacking *elf* exhibit increased numbers of cells in the developing cortex associated with loss of organization of developing cells. Neurosphere culture of dispersed cortical cells obtained from *elf* mutant mice results in small numbers of large primary neurospheres in culture compared to wild type. These results suggest that TGF- $\beta$  signaling is necessary for differentiation of precursor neuronal cells, while maintaining stem cell population without expansion. As a result, loss of TGF beta function prevents differentiation, but cortical precursor cells appear to be sustained. Others studies have demonstrated loss of cadherin and  $\beta$ -catenin intercellular adherence function in cells obtained from these animals. The appearance of the brain in *elf* KO mice is very similar to the brain of mice lacking  $\alpha$ -catenin [50]. Together with data that TGF- $\beta$  induces cell-cycle exit mediated by the cell cycle inhibitor p21 in rat embryo

brain slices [51], it appears safe to postulate that asymmetric cell division with differentiation of one of two daughter cells and maintenance of constant numbers of stem cell, is attributable to TGF- $\beta$  function.

### 5.1. Adult neurogenesis and TGF- $\beta$

Evidence suggests that adult neurogenesis occurs within an angiogenic niche in the hippocampus. Memory functions of the brain in the adult might correlate with adult neurogenesis. TGF $\beta$  appears to be involved in adult neurogenesis [53]. Transgenic mice over-expressing TGF- $\beta$ 1 produced virtually no new neurons in the hippocampus, and even at 9 weeks of age, TGF- $\beta$ 1 over expression caused a 60% decrease in the total number of immature neurons, hippocampal bromodeoxyuridine (BrdU) incorporation, and production of neurons and astrocytes. Cell fates of newly generated cells 1 day after BrdU labeling reveal that excess TGF- $\beta$ 1 does not alter the proportion of cells becoming neurons versus astrocytes. Transgenic mice that over-express TGF- $\beta$ 1 generate only 40% of the normal number of new cells, but the proportion of cells assuming each phenotype in TGF- $\beta$ 1 mice is not altered compared to wild-type mice.

## 6. Conclusion

Signals provided by proteins of the transforming growth factor (TGF)-beta family might represent a temporally and spatially active system by which neural stem cells are both supported but also induced into asymmetric division thus exiting the cell-cycle. Timed expression of membrane and intracellular signaling intermediates of TGF- $\beta$  adds a level of complexity to orchestration of the effect of this factor on development.

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