Mini Review

# Regulation of neural progenitor proliferation by EGF signaling in the spinal cord

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A wide variety of neurons and glial cells are generated in the developing central nervous system (CNS). The precise regulation of cell-type determination and cell number is thought to be indispensable for the appropriate development and physiological functions of the CNS. Both extrinsic and intrinsic molecular mechanisms, which regulate timing and position in cytogenesis, enable the acquisition of diversity and the control of numbers in neurons and glial cells. The specification of neurons and glial cells in the embryonic spinal cord has been intensively studied. Distinct types of neurons and glial cells are generated along the dorsoventral axis of the embryonic spinal cord, which is regulated by extracellular molecules and transcriptional codes. However, the molecular mechanisms regulating cytogenesis of certain progenitor cells through extracellular molecules have been poorly elucidated. We have recently demonstrated that epidermal growth factor (EGF) signaling via Grb2 associated binder1 (Gab1) contributes to the proliferation of Olig2-positive progenitors that sequentially generate motoneuron and glial cells in the developing spinal cord, in a time and position-restricted manner. In this review, the molecular mechanisms of progenitor proliferation via EGF signaling will be discussed by focusing on the embryonic spinal cord.

Rec.12/25/2007, Acc.1/15/2008, pp447-453

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**Key words** epidermal growth factor, Grb2 associated binder1, Olig2, proliferation, progenitor

## Introduction

Two main classes of cells comprise the central nervous system (CNS): neurons, the main signaling and memory units and glial cells, supporting cells. The human brain contains an extraordinary number of neurons (in the order of $10^{11}$) which are classified into at least a thousand different types[1]. Brain function depends on the specification of individual neurons and formation of precise anatomical circuits. Although glial cells are not directly involved in information processing, they are thought to play many vital roles. For example, astrocytes are involved in

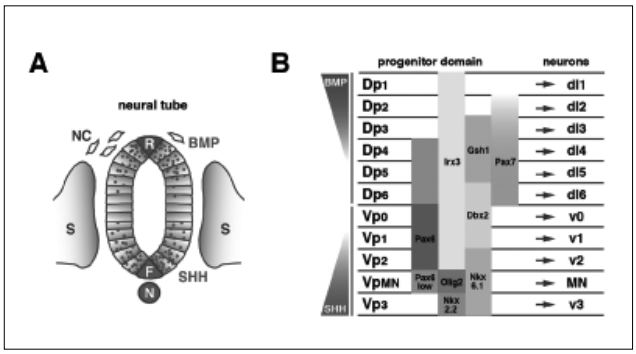


Fig.1 Extracellular molecules and neural subtype specification in the embryonic spinal cord along the dorsoventral (D-V) axis

(A) Sources of two classes of protein after neural tube closure which provide inductive signals are shown. Bone morphogenetic protein (BMP) is expressed in the roof plate (R) and in the adjacent neural tube and Sonic hedgehog (SHH) is secreted from the notochord (N) and floor plate (F). NC, neural crest; S, somite.

(B) Progenitor domains, where distinct combinations of basic helix-loop-helix (bHLH) and homeodomain transcription factors are expressed, are induced by graded BMP and SHH signals in the ventricular zones along the D-V axis of the embryonic spinal cord[13,48-50]. Subsequently, distinct classes of neurons are generated from each individual progenitor domain.

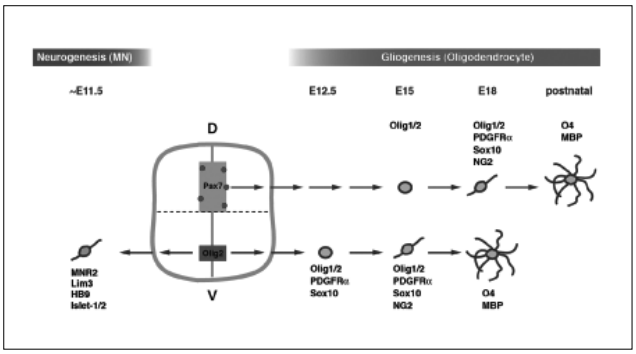


Fig.2 Sequential production of motoneurons and oligodendrocytes in the embryonic mouse spinal cord

Motoneurons (MN) are generated from the Olig2+ pMN domain by E11.5 in the mouse spinal cord (left). Oligodendrocytes are generated from the Olig2+ pMN domain after motoneuron generation (lower right). After E14.5, Olig2+ cells appear in the dorsal Pax7+ progenitor domain and differentiated into oligodendrocytes (upper right). Molecular markers in each developmental stage of cells are shown in black above or below[8,16].

the formation of the blood-brain barrier and control of the concentration of ions and neurotransmitters. Oligodendrocytes insulate individual axons by forming myelin in the vertebrate nervous system, so as to increase the transmission rate of nerve impulses. It has been thought that the ratio between neurons and glial cell numbers is likely to be of functional relevance[2] and the human brain contains between 10 and 50 times more glial cells than neurons[1]. The complexity of human behavior may involve the functions of many glial cells as well as that of individual neurons and their circuits. Both extrinsic and intrinsic regulatory mechanisms are required for the appropriate development of the nervous system in a context-dependent manner. Such complex regulatory mechanisms enable precise regulation of both specification and number of neurons and glial cells. As a mechanism regulating the number of cells, growth factors play crucial roles for the proliferation of neural progenitor cells, including stem cells, both *in vitro* and *in vivo*[3-6]. In this review, we focus on the signal transduction of epidermal growth factor (EGF) that is essential for the proliferation of Olig2-positive (Olig2+) neural progenitors in the developing spinal cord in a context-dependent manner.

## The specification of cell-types in the embryonic spinal cord

The specification of cell-types in the embryonic spinal cord has been extensively investigated. Genetic and embryological studies have elucidated a series of molecular mechanisms involving inductive signaling and transcriptional code. In the dorsoventral (D-V) axis within the neural tube, graded inductive signals such as bone morphogenetic proteins (BMPs) secreted from the roof plate and Sonic hedgehog (SHH) derived from the notochord and floor plate (Fig.1A), elaborate the precise pattern of expression of the homeodomain and basic helix-loop-helix (bHLH) transcription factors in progenitor domains located in the ventricular zone (VZ)[7-9]. Distinct types of cells are then generated from the progenitor domains defined by the combination of these transcriptional factors (transcriptional code) along the D-V axis (Fig.1B).

Olig2, for example, has been identified as a bHLH transcription factor expressed in the pMN domain under the control of the SHH signal[10,11]. Olig2 induces motoneurons in cooperation with another bHLH factor Neurogenin2 (Ngn2)[12,13], and subsequently specifies oligodendrocytes in combination with Nkx2.2[14,15]. More recently, it has been shown that a subpopulation of Pax7 (a paired homeodomain transcription factor) - expressing progenitor cells located in the dorsal half of the VZ of the mouse

embryonic spinal cord also starts to express Olig2 after embryonic day 14.5 (E14.5) and may subsequently differentiate into oligodendrocytes[16,17] (Fig.2). Analysis of mice lacking Olig2 has confirmed the essential role of Olig2 in the development of both motoneurons and oligodendrocytes[18,19].

## Regulation of progenitor proliferation by EGF signaling in the CNS

It has been shown to date that many kinds of growth factors are involved in CNS development. EGF/TGF$\alpha$ (transforming growth factor $\alpha$) and FGF-2 (fibroblast growth factor-2) are well-known mitotic signals for neural stem/progenitor cells (NSPCs) isolated from the embryonic and adult CNS including the spinal cord[4-6,20]. At the early stage of development, NSPCs are responsive to FGF-2 but not to EGF[21,22], then acquire responsiveness to EGF in the mid-gestational days[22,23]. The acquisition of responsiveness to EGF is associated with the appearance of a subpopulation of progenitor cells that express EGF receptor (EGFR) which is one of the typical receptor tyrosine kinases[21,24]. In the developing mouse spinal cord, EGFR is transiently expressed in the generative zone during the mid-gestational days at the peak of gliogenesis[23].

## Signal transduction pathways of EGFR

EGFR signaling has been investigated extensively in various tissues, and is mediated through signaling complexes consisting of many components[25]. Gab1 (grb2 associated binder1), originally identified as a Grb2 (growth factor receptor bound protein 2) binding-protein[26], is a member of the Gab/DOS (Daughter of sevenless) family of adaptor molecules. Gab1 contains a pleckstrin homology (PH) domain and binding sites for *src* homology 2 (SH2) and *src* homology 3 (SH3) domains, and plays a role as a common intracellular signaling mediator for various growth factors and cytokines, including EGFR, gp130 (a common receptor subunit for the IL-6 cytokine family), PDGFR (platelet-derived growth factor receptor), c-met (a receptor for HGF), Insulin-like growth factor-I receptor, and so on[27]. Depending on the stimulus of ligands, Gab1, which is recruited to activated receptors, assembles multimeric signaling complexes with Grb2, SHP2, p85 phosphatidylinositol 3-kinase (PI-3K), and phospholipase C-$\gamma$, and serves as a signal 'amplifier'[28] (Fig.3A). Those signals activate the Ras mitogen-activated protein kinase (Ras/MAPK) and PI-3K/Akt pathways mainly through the association of Gab1 in various tissues[29,30]. Thus, the preference for intracellular signaling molecules varies in different type of cells even in same ligand stimulus, suggesting that signal transduction downstream of EGFR depends on cellular contexts[31]. The detailed mechanisms of its regulations through the complex signal transduction pathways downstream of EGFR in the CNS remain to be elucidated.

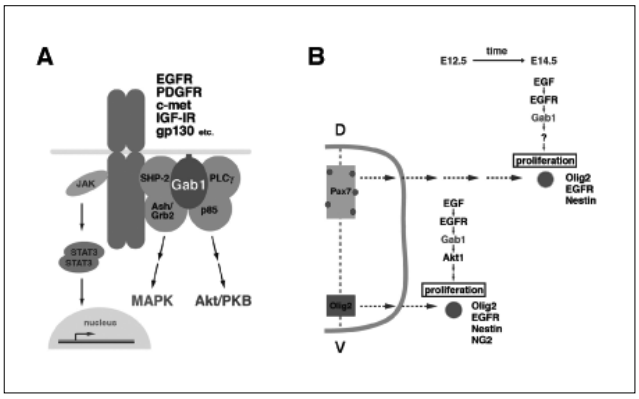


Fig.3 Schematic illustration of EGF/Gab1 signaling and its proliferative activity upon Olig2$^+$ progenitors in a time and position-restricted manner

(A) Gab1 (grb2 associated binder1) plays a role as a common signaling mediator for various growth factors and cytokines. After ligand binding to its receptors, Gab1 is recruited to activated receptors and assembles multimeric signaling complexes with Grb2, SHP2, p85 phosphatidylinositol 3-kinase (PI-3K), and phospholipase C-$\gamma$ (PLC$\gamma$). Subsequently, the Ras mitogen-activated protein kinase (Ras/MAPK) and PI-3K/Akt pathways are activated mainly through the association of Gab1. Signal transducers and activators of transcription 3 (STAT3) is activated through phosphorylation by mostly Janus kinases (JAKs) in various ligands including the IL-6 family of cytokines and growth factors.

(B) Gab1-deficient mice showed that EGF/Gab1 signaling is essential for the proliferation of Olig2$^+$ progenitors in the E12.5 ventral pMN domain and E14.5 dorsal Pax7$^+$ progenitor domain in a time and position-restricted manner. Additionally, Akt1 dependency downstream Gab1 may be variable in the cellular contexts. EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; IGF-I R, insulin-like growth factor I receptor.

## EGF/Gab1 signaling is essential for the proliferation of Olig2-positive progenitor cells

We have recently reported that Gab1-deficient (*Gab1*$^{-/-}$) mice exhibit the transient decrease of proliferation of Olig2$^+$ progenitors located at the pMN domain in E12.5-E13.5 mouse spinal

cord without any alteration in motoneuron development[32]. After E14.5 when oligodendrocytogenesis starts in the dorsal spinal cord, the number of Olig2$^+$ progenitors and Olig2/EGFR double positive progenitors derived from dorsal Pax7$^+$ progenitor domains also decrease. Surprisingly, EGF, but not FGF-2 or PDGF-AA, specifically maintains the mitotic state of Olig2$^+$ progenitors derived from the E12.5 ventral and E14.5 dorsal spinal cord *in vitro*. Gab1 deficiency resulted in the failure of this EGF-dependent proliferation of Olig2$^+$ progenitors. The expression of EGFR begins to be detected by immunohistochemistry predominantly in the Olig2$^+$ pMN domain of the E12.5- mouse spinal cord, when oligodendrocytogenesis starts after termination of mononeuron generation, and subsequently extends to the dorsal segment. This is consistent with the responsiveness of Olig2$^+$ progenitors to EGF and its failure in the E12.5 *Gab1*$^{-/-}$ spinal cord. However, in the case of the E14.5 spinal cord, EGF/Gab1 signaling contributes toward the proliferation of Olig2$^+$ progenitors only in the dorsal spinal cord, even though EGFR is expressed in both the ventral and dorsal spinal cord. Thus, EGFR/Gab1 signaling is utilized for Olig2$^+$ progenitor proliferation in a context-dependent manner and regulates its timing and position precisely in the developing spinal cord (Fig.3B). In addition, analysis of EGFR-deficient mice confirmed the specific contribution of the EGF signal to the proliferation of Olig2$^+$ progenitors *in vivo*.

## Downstream EGF/Gab1 signaling

Several pathways including the Ras/MAPK and PI3-K/Akt pathways have been shown to be activated downstream of EGFR via Gab1 in various tissues[33-35]. In the CNS, a small population of cells located in the VZ of the embryonic mouse cortex show high levels of expression of Akt1, and enhancement of Akt1 expression with a retrovirus vector resulted in an increase of phosphorylation of Akt1 at Thr-308 and in the number of EGF-responsive neurospheres[36]. Consistent with this, EGF-dependent activation of Akt1 (phosphorylation of Akt on Thr-308) was significantly reduced in the E12.5 whole or E14.5 dorsal spinal cord from *Gab1*$^{-/-}$ mice, whereas activation of ERK1/2 (p42/p44) was maintained. In contrast, loss-of-function studies showed reduction of EGF-induced MAPK activation in *Gab1*$^{-/-}$ MEFs[34] and *Gab1*$^{-/-}$ epidermis[33]. This discrepancy can be accounted for by the preferences for the signaling cascade in the different cell types even in the same ligand stimulation[31]. Alternatively, since it has been suggested that Gab1 mediated PI3K/Akt activation extends the duration of Ras/MAPK signaling[35], there may be differences in the signaling crosstalk between the MAPK and PI3K/Akt pathways downstream of Gab1.

The magnitude of the contribution of Akt1 to the EGF-dependent proliferation of Olig2$^+$ progenitors is also context-dependent. Interestingly, the expression of an active form of Akt1 using a lentivirus could rescue the proliferation of Olig2$^+$ progenitors in the E12.5 but not the E14.5 spinal cord of *Gab1*$^{-/-}$ mice. It has been shown that the balance of PI3K/Akt and MAPK determines the phenotypes of smooth muscle cells[37]. Therefore, an imbalance between the two signaling pathways rather than a mere reduction of the absolute level of active Akt1 might cause the defect of proliferation in the *Gab1*$^{-/-}$ mice. Alternatively, Olig2$^+$ progenitors from the E14.5 dorsal spinal cord might require unknown Gab1 targets other than Akt1 and MAPK for their EGF-dependent proliferation.

The signaling cascade of EGF via Gab1, therefore, varies even within the developing spinal cord in a time and position-restricted manner. Perhaps further studies using a series of knockin mice that carry mutation in binding motif of Gab1 to other component of multiple signaling complex will give us a more clear understanding of EGF/Gab1 signaling in the CNS.

## Regulation of EGFR expression and its function

It is thought that the expression of EGFR enables the acquisition of NSPC EGF-responsiveness in the mid-gestational days[22,23]. This expression of EGFR may be regulated precisely in their time and position. In the spinal cord, EGFR expression was detected in restricted the Olig2$^+$ pMN domain after E12.5, subsequently expanding into the dorsal region[32]. So far, it has been reported that the appearance of EGFR-expressing and EGF-responsive neural progenitors in the developing cortex is induced by FGF-2 and suppressed by BMP4[38]. BMPs are expressed in the roof plate of the neural tube[39], and expression of BMP receptors in the brain declines from the mid-embryonic stage[40]. This withdrawal of BMP receptors expression may allow EGFR expression to start in the ventral VZ of the E12.5 mouse spinal cord. Additionally, EGFR expression can be induced in the E12.5 mouse dorsal spinal cord cells *in vitro* by exposure to FGF-2[41]. These reports collectively suggest that at least FGF-2 and BMP4 as extrinsic factors are involved in the regulation of EGFR expression in the developing CNS. Moreover, intrinsic factors, such as transcription factors and epigenetic control, might play a role in the regulation of EGFR expression[42].

We have demonstrated that EGF maintains both the mitotic and undifferentiated state of Olig2$^+$ progenitors from the E12.5 whole and E14.5 dorsal spinal cord *in vitro*. Since these Olig2$^+$

progenitors behave just like NSPCs *in vitro*[43] and give rise to glial cells including oligodendrocytes after E12.5 *in vivo*[14,15], the spatiotemporal expression of EGFR could be important for the supply of sufficient amount of glial cells generated from NSPCs.

## Conclusions

Proliferation of various types of neural progenitors depends on the integration of multiple signaling pathways, including growth factors. Investigation of the roles of EGFR/Gab1 signaling in the developing mouse spinal cord revealed that the EGF/Gab1/Akt pathway plays essential roles in the proliferation of $Olig2^+$ progenitors in a spatiotemporally regulated manner and that utilization of Akt1 downstream EGF/Gab1 signaling for the proliferation of $Olig2^+$ progenitors varies in different biological contexts. These findings suggest that, in addition to the differential expression of ligands and receptors, differential utilization of intracellular signaling components is integrated into the regulation of progenitor proliferation to complete the CNS histogenesis by growth factor signals.

EGF signaling may also contribute to many biological aspects including the physiology and pathology of the adult CNS. In the SVZ (subventricular zone) lining the lateral ventricles, a site of adult neurogenesis, transit-amplifying cells express EGFR and generate neuroblasts that supply interneurons in the olfactory bulb[44,45]. Cerebral ischemia results in an increase of the number of the EGFR-positive transit-amplifying cells in the SVZ[46]. Moreover, amplification of the EGFR gene occurs in 40-50% of glioblastomas and the mutant receptor exerts an enhancement of tumorigenicity *in vivo*[47]. As described in this review, control of the progenitor proliferation might be underlain by a mechanism which is more sophisticated than we have thought. Further analyses of signal transduction on certain cellular contexts provide us with a deeper understanding of progenitor cell proliferation via EGF signaling in the adult neurogenesis and pathology as well as the development of the CNS.

### Acknowledgements

We apologize to authors whose works were not referenced due to limitations of space. We are grateful to Dr. Masato Yano for helpful comments and Ms. Akiyo Hirayama for preparing the MS. This study was supported by CREST/SORST-JST (H.O.), a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology in Japan (T.S. and H.O.), and a Grant-in-Aid for JSPS Fellows (Y.Y.).

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