Combinatorial actions of Tgfβ and Activin ligands promote oligodendrocyte development and CNS myelination

Dipankar J. Dutta1,2,3, Andleeb Zameer1,2,3, John N. Mariani1,2,3, Jingya Zhang1,2,3, Linnea Asp1,2,3, Jimmy Huyhn2,3,4, Sean Mahase1,2,3, Benjamin M. Laitman1,2,3, Azeb Tadesse Argaw1,2,3, Nesanet Mitiku1,2,3, Mateusz Urbanski5, Carmen V. Melendez-Vasquez5, Patrizia Casaccia2,3,4, Fernand Hayot1,3,6, Erwin P. Bottinger7,8, Chester W. Brown9,10 and Gareth R. John1,2,3,*

ABSTRACT

In the embryonic CNS, development of myelin-forming oligodendrocytes is limited by bone morphogenetic proteins, which constitute one arm of the transforming growth factor-β (Tgfβ) family and signal canonically via Smads 1/5/8. Tgfβ ligands and Activins comprise the other arm and signal via Smads 2/3, but their roles in oligodendrocyte development are incompletely characterized. Here, we report that Tgfβ ligands and activin B (ActB) act in concert in the mammalian spinal cord to promote oligodendrocyte generation and myelination. In mouse neural tube, newly specified oligodendrocyte progenitors (OLPs) are first exposed to Tgfβ ligands in isolation, then later in combination with ActB during maturation. In primary OLP cultures, Tgfβ1 and ActB differentially activate canonical Smad3 and non-canonical MAP kinase signaling. Both ligands enhance viability, and Tgfβ1 promotes proliferation while ActB supports maturation. Importantly, co-treatment strongly activates both signaling pathways, producing an additive effect on viability and enhancing both proliferation and differentiation such that mature oligodendrocyte numbers are substantially increased. Co-treatment promotes myelination in OLP-neuron co-cultures, and maturing oligodendrocytes in spinal cord white matter display strong Smad3 and MAP kinase activation. In spinal cords of ActB-deficient Inhbb−/− embryos, apoptosis in the oligodendrocyte lineage is increased and OLP numbers transiently reduced, but numbers, maturation and myelination recover during the first postnatal week. Smad3−/− mice display a more severe phenotype, including diminished viability and proliferation, persistently reduced mature and immature cell numbers, and delayed myelination. Collectively, these findings suggest that, in mammalian spinal cord, Tgfβ ligands and ActB together support oligodendrocyte development and myelin formation.

KEY WORDS: Transforming growth factor, Activin, Smad, Oligodendrocyte, Myelin

INTRODUCTION

Within CNS white matter, oligodendrocytes form myelin sheaths that facilitate efficient nerve impulse transmission (Li et al., 2009). Their origins have been studied in developing spinal cord, and oligodendrocyte progenitors (OLPs) are known to be produced in two phases (Richardson et al., 2006). The first occurs ventrally in the motor neuron progenitor (pMN) domain of periventricular neuroepithelium (Orentas and Miller, 1996) in response to sonic hedgehog induction of Olig transcription factors (Lu et al., 2000). The second phase is more dorsal (Cai et al., 2005) and depends on inhibition or downregulation of roof plate-derived bone morphogenetic proteins (Bmps), which restrict lineage specification and maturation (Mekki-Dauriac et al., 2002; Miller et al., 2004). Following specification, OLPs proliferate and migrate within the parenchyma, then differentiate into myelinating cells in white matter tracts (Fancy et al., 2011).

Bmps comprise one arm of the transforming growth factor-β (Tgfβ) family and signal canonically via R-Smads 1/5/8 (Munoz-Sanjuan and Brivanlou, 2002). The other arm includes Tgfβ1-5 and Activins, which signal via Smads 2/3 (Shi and Massague, 2003). Notably, the roles of this Smad2/3-activating cohort in oligodendrocyte development remain incompletely characterized. Complementary patterns of Tgfβ1-3 expression have been reported in embryonic mouse spinal cord (Flanders et al., 1991; Mecha et al., 2008), and Tgfβ1 has been shown to regulate Pdgf-driven proliferation of O-2A cells in vitro (McKinnon et al., 1993), while interactions between Olig2 and Smad3 have been linked to boundary determination of the pMN domain at HH stages 12-18 in chick neural tube (Garcia-Campmany and Marti, 2007; Estaras et al., 2012). Studies have also reported expression of activin B (ActB) subunit and ActrIIb receptor mRNAs in developing mouse CNS at stages including those of OLP generation (Feijen et al., 1994). These findings are further compatible with known roles of Activins as regulators of neuronal survival and differentiation (Schubert et al., 1990; Poulsen et al., 1994; Kriegstein et al., 1995; Cambray et al., 2012).

Here, we report that, in contrast to Bmp signaling, Tgfβ ligands and ActB act in combination in the developing mammalian spinal cord to promote oligodendrocyte development and myelination. Our expression analyses in mouse embryos indicate that, following lineage specification, OLPs are exposed to Tgfβ ligands first alone, then in combination with ActB during maturation. In isolation, Tgfβ ligands and ActB differentially activate canonical Smad3 and non-canonical MAP kinase signaling. Both enhance viability, and Tgfβ ligands promote proliferation whereas ActB enhances differentiation. In combination, they strongly activate both signaling pathways. Together, their impact on viability is additive, and proliferation and differentiation are both enhanced, resulting in a substantial increase in mature oligodendrocyte numbers. In ActB-deficient Inhbb−/− mouse embryos, apoptosis is increased within the lineage, and OLP numbers are transiently reduced. Notably, Smad3−/− embryos display more severe defects in viability, mitosis and maturation, and these result in delayed myelination.
RESULTS
Localization of Smad3 activity in E12.5 mouse spinal cord
We analyzed embryonic mouse spinal cord for patterns of canonical Smad2/3 activity, and correlated these with expression profiles of Tgfβ and Activin ligands (Figs 1 and 2; supplementary material Fig. S1). Notably, these immunohistochemical studies revealed phosphorylated (active) Smad3, but not Smad2, in oligodendrocyte lineage cells from embryonic day (E) 12.5, shortly after ventral OLP specification (Fig. 1; supplementary material Fig. S1). At E12.5, Smad3 protein and phospho-Smad3 (P-Ser423/425) were detected in cell nuclei throughout the periventricular neuroepithelium (Fig. 1A-C), including Olig2+ OLPs in the pMN domain (Fig. 1A; supplementary material Fig. S1A). Total and phospho-Smad3 also localized to Lim1+ Dl1/Dl2 neurons dorsolaterally in the parenchyma, and to the floor plate ventrally (Fig. 1A; supplementary material Fig. S1A,B). No immunoreactivity was detected in Smad3−/− embryos (negative control). These findings were similar, but not identical, to previous reports in chick, in which Smad3 is expressed in periventricular neuroepithelium but is relatively low in the pMN domain at the equivalent stage (Garcia-Campmany and Marti, 2007; Estaras et al., 2012). Compatible with previous findings, Smad2 was not detected (Garcia-Campmany and Marti, 2007). Reports differ as to the extent of Smad2 expression in the neural tube during embryogenesis (Garcia-Campmany and Marti, 2007; Miguez et al., 2013).

Differential patterns of Tgfβ and Activin ligands in mouse spinal cord at E12.5
Further analyses suggested that in developing spinal cord, Smad3 activity might first result from exposure to Tgfβ1-3 in periventricular neuroepithelium, then to these and ActB together in the

Fig. 1. Smad3 activation in E12.5 mouse spinal cord. Spinal cord sections from E12.5 C57BL/6 embryos immunolabeled for Smad3 and Tgfβ and Activin ligands and receptors. (A-C) Phospho-Smad3 (P-Ser423/425) was detected throughout the Sox2+ periventricular neuroepithelium (A,B), which is proliferating (Ki67+) (C). See also supplementary material Fig. S1A. Olig2+ OLPs in the pMN domain were P-Smad3+ (A, white arrow and inset). P-Smad3 also localized to Lim1+ Dl1/Dl2 neurons dorsolaterally in the parenchyma, and to the floor plate (arrowhead). V, ventricle. (D-G) Expression of Tgfβ1-3 and Tgfβr2. Olig2+ cells are arrowed and magnified in the inset. Similar to P-Smad3, Tgfβ1 localized extracellulary to Sox2+ periventricular neuroepithelium including the pMN domain, and to Lim1+ neurons dorsolaterally (D). See also supplementary material Fig. S1B. Tgfβr2 localized to cells throughout the spinal cord, including Olig2+ OLPs (E). Tgfβ2 displayed a similar distribution to Tgfβ1 (F), whereas Tgfβ3 localized extracellularly to ventral periventricular zone and floorplate (G, arrowhead), and to the ventrolateral motor neuron domain (blue arrow). (H,I) Distribution of ActB and ActrIIb. ActB was detected extracellularly within the Mnr+ Isl2+ ventrolateral motor neuron domain (H, blue arrow and inset). See also supplementary material Fig. S1C,D. ActrIIb was expressed by cells throughout the periventricular neuroepithelium and dorsolateral cord, including Olig2+ OLPs (I, arrow and inset). Data are representative of samples from at least four individuals. Scale bars: 50 µm.
parenchyma. Tgfβ1 has highest affinity for the ligand-binding
TgfβR2 receptor (Shi and Massague, 2003) and, compatible with
Smad3 activity and a previous report (Mecha et al., 2008), at E12.5
Tgfβ1 protein localized extracellularly throughout periventricular
neuroepithelium, and to the Lim1+ neuronal domain (Fig. 1D;
supplementary material Fig. S1B). TgfβR2 localized to cells
throughout the spinal cord, including Olig2+ OLPs (Fig. 1E). We
also detected the lower affinity ligands Tgfβ2 and Tgfβ3, with
the former localizing extracellularly to the periventricular
neuroepithelium and dorsolaterally (Fig. 1F), and the latter to the
ventral periventricular zone and floorplate and the ventrolateral
motor neuron domain (Fig. 1G). Thus, at E12.5 Olig2+ OLPs
were exposed to Tgfβ1-3, expressed TgfβR2, and were positive for
Smad3 activity.

By contrast, at E12.5 ActB localized extracellularly to the
Mnr+ Isl2+ ventrolateral motor neuron domain in the parenchyma
(Fig. 1H; supplementary material Fig. S1C,D). Activins A, B
and AB are dimers of Inhβa and Inhβb, and in situ hybridization
for Inhbb produced identical findings (supplementary material
Fig. S1C,D). Samples from Inhbb−/− embryos produced no
signal. The ligand-binding receptor ActrIIb localized throughout
the periventricular neuroepithelium and dorsolateral cord
(Fig. 1I), in agreement with a previous report (Feijen et al.,
1994). We did not detect ActrIIb localization to the Mnr− Isl2−
domains, the sites of ActB expression (Fig. 1I). Compatible with
previous findings, ActA and Inhba were not detected (Feijen
et al., 1994).

These data suggested that Tgfβ ligands are a significant source
of Smad3 activity at E12.5 throughout the periventricular
neuroepithelium, including Olig2+ OLPs. Although these cells
also expressed ActrIIb, at E12.5 the source of ActB lay outside the
periventricular zone, in the ventrolateral Mnr− Isl2− domain.

Fig. 2. Progressive overlap of Tgfβ1 and ActB during spinal cord development. (A,D,F) C57BL/6 spinal cords at E14.5, E16.5 and P5. In each, the left panel shows half a spinal cord immunolabeled for Tgfβ1 and Olig2, whereas the right panel illustrates immunoreactivity for ActB and Isl2 or NeuN. Representative (boxed) dorsal and ventral areas are shown at higher magnification in B,E,G. The central zone in A (arrowed) is shown at higher magnification in C in the case of Tgfβ1/Olig2 (left panel). The right panel in C shows ActB/Olig2 staining in the same area from an immediately adjacent section. By E14.5, Tgfβ1 expression began to expand ventrally within the parenchyma, such that it began to overlap with Olig2+ cells outside the periventricular neuroepithelium (A−C). Ventrally spread
continued during later embryogenesis (E16.5; D,E), and postnatally. By P5, when the spinal cord is myelinating, Tgfβ1 localized to neurons throughout the cord (F,G). Concurrently, ActB expression spread dorsally and medially. At E14.5, the pattern of ActB overlapped minimally with Olig2+ cells (A−C). By E16.5, ActB localized more widely throughout the parenchyma (D,E), and by P5 neuronal expression was widespread (F,G). Data are representative of four individuals at each time point. Scale bars: 70 µm in A; 15 µm in B,C; 150 µm in D; 30 µm in E; 250 µm in F; 60 µm in G.
Progressive overlap of Tgfβ1 and ActB during spinal cord development

Notably, these patterns subsequently changed, reflecting progressive overlap of Tgfβ and ActB signaling (Fig. 2; supplementary material Figs S1 and S2). This was detectable by E14.5, at which time OLPs expand into the parenchyma (Cai et al., 2005; Fogarty et al., 2005; Zhang et al., 2006). Coincident with this expansion, the distribution of Tgfβ1 spread ventrally, such that it began to overlap with Olig2+ cells outside the periventricular neuropil (Fig. 2A-C). The distribution of Tgfβ2 also expanded ventrally (supplementary material Fig. S1E), but Tgfβ1 remained confined to the periventricular zone, floorplate and ventrolateral parenchyma (supplementary material Fig. S1F). Ventral spread of Tgfβ1 continued during later embryogenesis (E16.5, Fig. 2D,E) and postnatally. By postnatal day (P) 5, when the spinal cord is myelinating, Tgfβ1 localized to neurons throughout the cord (Fig. 2F,G).

Concurrent with this increase in the distribution of Tgfβ ligands, ActB also spread dorsally and medially, albeit lagging behind the dorsal expansion of OLPs at E14.5 (Fig. 2A-C). By E16.5, ActB localization encompassed neuronal domains centrally and dorsally (Fig. 2D,E), and this trend continued until, by P5, neuronal expression was widespread (Fig. 2F,G). Thus, by the onset of myelination, Tgfβ1 and ActB overlapped almost completely.

Indicating that the oligodendrocyte lineage remained sensitive to Tgfβ and Activin ligands postnatally, at P5 both Tgfβ1 and ActB were expressed in Olig2+ cells, including Pdgfrα+ OLPs and mature CC-1+ oligodendrocytes, as well as to other lineages, including NeuN+ neurons (supplementary material Fig. S2A-F).

Together, these data suggested that OLPs in the developing spinal cord are initially exposed to Tgfβ ligands, then to these and ActB together. This suggested the potential for complementary functions and additive or synergistic outcomes.

Distinct signaling profiles produced by Tgfβ1, ActB, and co-treatment in OLPs in vitro

We compared the impact of Tgfβ1 and ActB individually and in combination in primary OLPs and the oligodendrocyte-derived Oli-Neu cell line (Figs 3 and 4; supplementary material Figs S3 and S4). Immunocytochemistry confirmed ubiquitous Tgfβ1 and ActB expression in OLPs (supplementary material Fig. S3A) and Oli-Neu cells (not shown). Initially, we evaluated the activation of canonical and non-canonical signaling in response to 1-100 ng/ml Tgfβ1, ActB, and co-treatment, in timecourse studies in Oli-Neu cells (supplementary material Fig. S3B-K), validated in primary OLPs (Fig. 3A,B). Although Act/Tgfβ ligands canonically activate Smad2/3, they also signal non-canonically via the p38/Erk/Jnk MAP kinase and PI3 kinase-Akt-Gsk3 pathways (Massague, 2012). Notably, examination of Smad3 and MAP kinase and Akt-Gsk3 activation profiles revealed that Tgfβ1 and ActB produced different patterns of canonical and non-canonical signaling, and that, crucially, in combination they elicited a third distinct response.

In both sets of cultures, Tgfβ1 and ActB both induced Smad3 phosphorylation at Ser423/425, but ActB produced a stronger, more prolonged effect than the same Tgfβ1 concentration (supplementary material Fig. S3B,C; Fig. 3A,B). ActB also reduced activation (phosphorylation) of p38 (P-Thr180/Tyr182) and p42/44 (P-Thr202/Tyr204) MAP kinases, which has been linked to reinforcement of Smad activation (Kretzschmar et al., 1999; Grimm and Gurdon, 2002). By contrast, Tgfβ1 briefly increased MAP kinase activation (supplementary material Fig. S3B,D; Fig. 3A,B). Importantly, co-treatment with ActB plus Tgfβ1 produced levels of phospho-Smad3 similar to, or greater than, ActB alone, and increased p38 and p42/44 phosphorylation beyond Tgfβ1 alone at later time points (30-60 min) (supplementary material Fig. S3B-D; Fig. 3A,B). Total Smad3 and MAP kinase protein levels were unaltered (supplementary material Fig. S3B,E,F). No activation of c-Jun (P-Ser63/73) was detected (data not shown). Neither ligand alone, nor in combination, impacted Akt (P-Thr308) or Gsk3α/β (P-Ser21/9) activity (supplementary material Fig. S3B,G-I).

Analysis of other regulatory mechanisms reported as Tgfβ1/ActB-sensitive in other cell types revealed no further differential outcomes. Smad3 phosphorylation within its linker region at Ser208 has been shown to alter its transcriptional activity (Kretzschmar et al., 1999; Funaba et al., 2002), but we detected no changes in Smad3 (P-Ser208) activity (supplementary material Fig. S3B,J). Additionally, we used co-immunoprecipitation to examine Smad3 interactions with potential co-activators, with Foxh1 serving as a positive control (Chen et al., 1996; Attisano et al., 2001). However, although we detected Smad3 association with Foxh1, we did not detect interactions with other co-activators, including ETF (Tead2), Sp1, Gli, TCF (Hnf4α) and Foxo1, nor any differential Smad3 binding to Foxh1 upon ActB or Tgfβ1 treatment (supplementary material Fig. S3K,L).

Interestingly, Tgfβ1 and ActB signaling were subject to regulation by Bmp. OLPs expressed Bmpr2 (supplementary material Fig. S3A), and, in agreement with previous work (De Robertis and Kuroda, 2004), in OLP cultures Bmp4 dose-dependently induced phosphorylation of Smad1/5 (P-Ser463/465) but not Smad3 or MAP kinases (Fig. 3A,B; supplementary material Fig. S4A-D). However, Bmp4 dose-dependently abrogated ActB- or Tgfβ1-induced Smad3 phosphorylation, but ActB or Tgfβ1 was unable to inhibit Bmp4 Smad1/5 activation (supplementary material Fig. S4A-D).

Co-treatment of OLPs with Tgfβ1 and ActB elicits combinatorial functional outcomes

These data indicated that exposure of OLPs to Tgfβ ligands in isolation versus in combination with ActB produced differential signaling profiles. Importantly, further analyses confirmed that these correlated with distinct functional outcomes (Fig. 3C-J; supplementary material Fig. S4E-G). In primary OLP cultures grown for 5 days in serum-free media, proliferating OLPs exit the cell cycle and mature into Cnp+ Mbp+ oligodendrocytes (Zhang et al., 2011). Initial immunoblotting studies revealed that treatment of cultures with Tgfβ1 or ActB alone or in combination reduced caspase-3 cleavage (reflecting apoptotic activity), and that ActB or co-treatment also enhanced maturation marker expression (Fig. 3C,D). In more comprehensive morphometric timecourse studies, both Tgfβ1 and ActB reduced the number and fraction of apoptotic cells (Fig. 3E,F; supplementary material Fig. S4E). In addition, Tgfβ1 increased numbers of Olig2+ oligodendrocyte lineage cells, and the number and fraction of proliferating BrdU-labeled Olig2+ cells, and of immature cells expressing Olig2 but not maturation markers (Fig. 3G-I; supplementary material Fig. S4F).

By contrast, ActB increased the number and fraction of mature Mbp+ oligodendrocytes (Fig. 3J; supplementary material Fig. S4G). Crucially, co-treatment produced a combination of these outcomes. Co-treatment reduced apoptosis more strongly than Tgfβ1 or ActB, and increased Olig2+ and BrdU− Olig2+ cells similar to Tgfβ1 alone (Fig. 3F-H; supplementary material Fig. S4E,F). Importantly, co-treatment also strongly potentiated the impact of ActB on mature cell number. In co-treated cultures, the fraction of Mbp+ cells was similar to that in ActB-treated cultures (supplementary material Fig. S4G), but the overall Olig2+ population was significantly larger (Fig. 3H); thus, the total increase in Mbp+ cells was almost double that seen in cultures treated with ActB alone (Fig. 3J). Different markers for
**Fig. 3.** See next page for legend.
Fig. 3. Tgfβ1, ActB and co-treatment elicit distinct signaling and functional outcomes in OLPs. (A-D) Immunoblotting (A,C) and densitometry (B,D) of primary OLPs plated into serum-free media and exposed to 50 ng/ml Tgfβ1, ActB and/or Bmp4 for 30 min (A,B) or 5 days (C,D). (A,B) Results complement signaling timecourse data from Oli-Neu cells (supplementary material Fig. S3A-L) and from OLPs (supplementary material Fig. S4A-D). (C,D) Data accompany morphometric timecourse analyses in OLPs in E-J and supplementary material Fig. S4E-G. (A,B) In OLPs, Tgfβ1 and ActB both induced Smad3 phosphorylation (P-Ser423/425), but ActB produced stronger stimulation than the equivalent Tgfβ1 concentration. ActB also reduced phosphorylation of p38 (P-Thr180/Tyr182) and p42/44 (P-Thr202/Tyr204). Co-treatment produced Smad3 phosphorylation similar to, or greater than, ActB alone, without inhibition of MAP kinase phosphorylation; rather, co-treatment increased phospho-p38 (P-Thr180/Tyr182) and p42/44 (P-Thr202/Tyr204). See also supplementary material Fig. S3A-F. Conversely, Bmp4 induced phosphorylation of Smads 1/5 (P-Ser463/465) but not Smad3. Co-treatment with Tgfβ1 or ActB plus Bmp4 abrogated Smad3 phosphorylation but not Smad1/5/8 phosphorylation. See also supplementary material Fig. S4A-D. (C,D) At 5 days, caspase-3 cleavage (a marker of apoptotic activity) was decreased in OLP cultures exposed to Tgfβ1 or ActB alone or together. Mbp expression (mature oligodendrocytes) was enhanced in ActB-treated and co-treated cultures. Bmp alone or in combination with Tgfβ1 or ActB abrogated Mbp expression. (E-J) OLPs grown in serum-free media and treated with 50 ng/ml Tgfβ1, ActB or both were harvested at 12 h intervals for up to 5 days and stained for Olig2, Mbp, cleaved caspase-3 and BrdU labeling (proliferation) (E). Data are presented as raw cell counts. The same results are presented as fractions of total Olig2+ cells in supplementary material Fig. S4E-G. In controls, mature Mbp+ cells progressively increased, proliferating progenitors decreased, and apoptosis gradually increased. Tgfβ1 or ActB reduced numbers of apoptotic cells (F). Tgfβ1 also amplified numbers of proliferating BrdU+ Olig2+, total Olig2+ and immature Olig2+ Mbp+ cells (G-I), whereas ActB increased mature Mbp+ oligodendrocytes (J). Co-treatment reduced apoptotic cell number more substantially than either ligand alone, and increased BrdU+ Olig2+ and total Olig2+ cells similar to Tgfβ1 (F-H). Co-treatment also potentiated the impact of ActB on mature Mbp+ oligodendrocyte number. In co-treated cultures, the fraction of Mbp+ cells was similar to that in ActB-treated cultures (see supplementary material Fig. S4G), but the Olig2+ population was larger, and thus Mbp+ cell number was strongly augmented (J). Data are representative of three studies in separate cultures. (F-J) Two-way ANOVA plus Bonferroni post-test; *P<0.05, **P<0.01, ***P<0.001, for co-treated versus vehicle (Veh) control cultures. Error bars indicate s.e.m.

Immature versus mature cells (OLP, Pdgfα; oligodendrocyte, Plp) produced identical findings.

Tgfβ1 and ActB synergistically promote oligodendrocyte maturation and enhance viability
To validate these outcomes, from morphometric data we used a previously described mathematical model to calculate coefficients for proliferation in the Olig2+ Mbp+ (OLP plus immature oligodendrocyte) versus Olig2+ Mbp+ (mature) populations (α, γ), differentiation from one to the other (β), and net apoptosis (δ+ε) (Zhang et al., 2011; Hsieh et al., 2004) (Fig. 4A). Coefficients thus generated take into account rate of change of the measured parameters and population size. ActB and Tgfβ1 each reduced the coefficient of apoptosis (Fig. 4B), and Tgfβ1 increased the coefficient of proliferation (Fig. 4C), whereas ActB enhanced the coefficient of differentiation (Fig. 4D). All values for γ were zero, confirming the postmitotic status of Mbp+ cells (not shown). Importantly, co-treatment produced a further reduction in the apoptosis coefficient (Fig. 4B), combined with strong enhancement of the differentiation coefficient (Fig. 4D).

Strong Smad3 and MAP kinase activity in differentiating oligodendrocytes in myelinating white matter
To test whether these in vitro profiles were also reflected in vivo, we returned to analyses of developing spinal cord (Fig. 5). OLPs in vivo are exposed to Tgfβ1 ligands first in isolation, and then in combination with ActB during maturation. Thus, we hypothesized that Smad3 and MAP kinase activity would be detectable in OLPs following specification and during expansion, and then increase in differentiating cells. A similar pattern has been described for Erk signaling in chick, with activity most pronounced in differentiating white matter (Kato et al., 2005). To test the hypothesis, we measured pixel intensity for Smad3 (P-Ser423/425) and p42/44 (P-Thr202/Tyr204) in cells positive for lineage or maturation markers at E12.5, during expansion at E14.5, and at the onset of myelination at P5. Smad3 activity could be assumed to result from Tgfβ1/Activin signaling. MAP kinase pathways have been discussed in the context of OLP development (Schwannenthal et al., 2004; Fragosco et al., 2007; Fyffe-Maricich et al., 2011), and are also activated by factors beyond the Tgfβ1 family (Bhat and Zhang, 1996; Althaus et al., 1997; Yim et al., 2001).

Quantitative analysis confirmed detectable Smad3 (P-Ser423/425) activity in OLPs in the pMN domain at E14.5, as at E12.5 (Fig. 5A,B; compare with Fig. 1A). Activity was also detectable in Olig2+ cells migrating into the parenchyma at E14.5, albeit at lower levels (Fig. 5A,B), consistent with the lower Tgfβ1 and Tgfβ2 activity in these areas at this time (compare with Fig. 2A). Notably, however, by P5 we observed a differential in Smad3 activity (Fig. 5B,C), which was high in Olig2+ cells in maturing white matter and lower in Olig2+ cells in gray matter (Fig. 5B,C). Further supporting our hypothesis, cells positive for maturation markers (CC-1) also displayed high-level activity in P5 white matter tracts (Fig. 5C).

Similar results were also observed for p42/44 activity, which in addition were similar to those reported in chick (Kato et al., 2005). At E12.5, p42/44 (P-Thr202/Tyr204) localized to cells in the ventral periventricular neuroepithelium, including OLPs (Fig. 5D,E), and to radial processes between this region and the pial surface (Fig. 5E). A similar pattern was seen at E14.5 (Fig. 5F,D), and levels of p42/44 activity in Olig2+ cells in the pMN domain resembled those at E12.5 (Fig. 5D-F). Conversely, activity was detectable but low in Olig2+ cells in the parenchyma at E14.5 (Fig. 5F,D). By P5, p42/44 (P-Thr202/Tyr204) localized predominantly to white matter, and to neurons within the dorsal horns (Fig. 5G). Activity was high in Olig2+ cells and CC-1+ cells in white matter tracts (Fig. 5D,G).

Together, these data suggested that the signaling profiles detected in response to Tgfβ1 and ActB treatment of OLPs in vitro are also reflected in the developing spinal cord, and are functionally relevant in the context of oligodendrocyte development. Consistent with our hypothesis, Smad3 and MAP kinase activity were both high in differentiating cells in white matter at P5, at the onset of myelination.

Co-treatment with Tgfβ1 plus ActB enhances myelin formation in OLP-neuron co-cultures
To investigate roles in myelin formation, we initially examined co-cultures of primary OLP and dorsal root ganglion (DRG) neurons (Fig. 6; supplementary material Fig. S5) (Chan et al., 2004). Co-cultures were left to myelinate in the presence of 50 ng/ml Tgfβ1, ActB, or both for 14 days, then stained for Olig2, Mbp and neurofilament (NF, axons) and analyzed by morphometry. We also examined the impact of co-treatment with Bmp4, which studies in OLPs confirmed as a potent inhibitor of maturation (supplementary material Fig. SSA-D) (See et al., 2004).

Importantly, these experiments showed that myelination was enhanced by ActB and further increased by Tgfβ1 plus ActB co-treatment. At 14 days, controls and ActB-treated or Tgfβ1-treated co-cultures contained Mbp+ oligodendrocytes, which
Extended processes that co-localized with NF axons to form linear Mbp+ NF profiles corresponding to compact myelin segments (Fig. 6A,B), as confirmed by electron microscopy (Fig. 6C). Co-cultures exposed to ActB contained more Mbp+ cells and myelin segments than controls (Fig. 6D,E). Tgfβ1 alone did not enhance myelination, but, importantly, Tgfβ1 plus ActB co-treatment resulted in a further increase in Mbp+ cells and segment numbers (Fig. 6A,D,E), also accompanied by an increase in myelination per Mbp+ cell (Fig. 6F). By contrast, co-cultures exposed to Bmp4 contained almost no Mbp+ cells or myelin segments, even in the presence of ActB co-treatment (Fig. 6D,E,G).

**Transient impairment of oligodendrocyte development in ActB-deficient Inhbb−/− spinal cord**

To investigate functional significance *in vivo*, we examined oligodendrocyte development and myelination in spinal cords of ActB-deficient Inhbb−/− and Smad3-deficient Smad3+/−xox8(Smad3−/−) mice (Vassalli et al., 1994; Yang et al., 1999) (Figs 7 and 8; supplementary material Fig. S6). Constitutive models were preferred since they allowed inference of wider trends within the cord. These studies revealed that oligodendrocyte development was abnormal in both models, but that the phenotype of Smad3−/− mice was the more severe.

In Inhbb−/− embryos, oligodendrocyte development was transiently impaired, but recovered postnatally and myelination was not impacted (Fig. 7). At E12.5, differences in Olig2+ cells did not reach significance (Fig. 7A,B). However, Olig2+ numbers were reduced in Inhbb−/− embryos at E15.5, a time when their distribution begins to overlap with that of ActB (Fig. 7C,D). At E15.5, Olig2+ Sox2− Sox9+ progenitors are found in the parenchyma, and early-stage Olig2+ Sox2+ progenitors remain in the periventricular zone (Stolt et al., 2003). In Inhbb−/− embryos, the former were decreased, but no changes were detected in the latter (Fig. 7C,D). We hypothesized that the former population might be more sensitive to changes in ActB support. Cleaved caspase-3+ Olig2+ apoptotic cells were increased in E15.5 Inhbb−/− samples (Fig. 7E), but cycling Ki67+ Olig2+ cells were unaltered (Fig. 7F).

Interestingly, this phenotype self-corrected postnatally. Multiple pathways contribute to the generation of myelinating cells, and a strong precedent exists for recovery of myelination in mutants showing deficits in progenitor development (Montag et al., 1994; Larsen et al., 2006; Lewallen et al., 2011). Postnatally in Inhbb−/− spinal cords, the fraction of apoptotic Olig2+ cells remained slightly but significantly increased, and the fraction of cycling Ki67+ Olig2+ cells was marginally (but significantly) raised (Fig. 7E,F), but by P5 the numbers of Olig2+ cells in Inhbb−/− and control cords were similar (Fig. 7G-I). No significant differences were detected in the numbers of Olig2+ Pdgfrα OLPs or in more mature CC-1+ oligodendrocytes (Fig. 7G). Cells expressing the early myelin protein Mag (myelin-associated glycoprotein) were reduced, but immunoreactivity for other myelin proteins was normal (Fig. 7G-I).

These findings suggested that loss of ActB resulted in a transient reduction in Olig2+ cell viability in Inhbb−/− embryos, and were also compatible with a delay in maturation. Although subtle effects persisted postnatally, this phenotype largely resolved, such that myelination was not delayed.

**Delayed myelination in spinal cords of Smad3−/− mice**

Importantly, examination of Smad3−/− mice revealed a more severe oligodendrocyte phenotype, which included delayed myelination (Fig. 8). At E12.5, Olig2+ cells and Sox2+ cells were both reduced in Smad3−/− spinal cords (Fig. 8A,B). Olig2+ cells remained diminished at E15.5, encompassing both the parenchymal Olig2+ Sox2− Sox9+ and periventricular Olig2+ Sox2+ populations (Fig. 8C,D). This reduction persisted at P5 in Smad3−/− spinal cords (Fig. 8E-G), and included both Olig2+ Pdgfrα OLPs and more mature CC-1+ oligodendrocytes (Fig. 8E). Mag+ cells were also decreased, and expression of Mbp and other mature myelin markers was impaired (Fig. 8E-G). These changes were associated with decreased viability and proliferation. Cleaved caspase-3+ Olig2+ apoptotic cells were substantially increased in Smad3−/− spinal cords, and cycling Ki67+ Olig2+ cells were slightly, but significantly, reduced at E12.5 and P5 (Fig. 8H,I). No compensatory
Induction of Smad2 was observed. No abnormalities were detected in Smad3+/- heterozygotes (not shown). Interestingly, this phenotype also eventually self-corrected, although compensation was delayed. By P28, no differences were detected in Olig2+ or Mag+ cell numbers or in Mbp immunoreactivity in spinal cords of Smad3−/− mice versus controls (Fig. 8J,K).

Together, these results demonstrated that loss of Smad3 signaling in vivo resulted in a prolonged (but not permanent) reduction in Smad3 activity.
oligodendrocyte lineage cells that was associated with persistently impaired viability and proliferation, decreased numbers of progenitors and mature cells, and delayed myelination.

Tgf\(\beta\)1 and ActB promote neural progenitor viability in vitro

Since the phenotype of Smad3\(^{-/-}\) mice included effects on Sox2\(^{+}\) progenitors, we examined outcomes of Tgf\(\beta\)1 treatment in primary neural progenitor cultures (supplementary material Fig. S6). ActB was included for comparison, although it does not overlap the periventricular zone in early embryogenesis. Adherent cultures were grown in media favoring proliferation (containing Egf) or specification and differentiation (growth factor-free), in the presence of 1-100 ng/ml Tgf\(\beta\)1, ActB or vehicle (Conti et al., 2005; Louis et al., 2013). Cells positive for the neural progenitor marker nestin, Olig2, caspase cleavage and BrdU-labeling were quantified at 12 h and 60 h in proliferation media and at 96 h in differentiation media.

Notably, both Tgf\(\beta\)1 and ActB improved neural progenitor viability in these studies, in the absence and in the presence of Egf. ActB and Tgf\(\beta\)1 both reduced the numbers and fraction of cleaved caspase-3\(^{+}\) cells under both culture conditions (supplementary material Fig. S6A,B). However, Egf has been reported as crucial for neural progenitor proliferation, and neither Tgf\(\beta\)1 nor ActB impacted the BrdU\(^{+}\) fraction in Egf-containing media (supplementary material Fig. S6C) nor was able to rescue proliferation in Egf-free media (supplementary material Fig. S6D). In addition, neither ligand affected oligodendrocyte lineage specification. No Olig2\(^{+}\) cells were observed in proliferation medium under any condition, with the population remaining entirely nestin\(^{+}\) (supplementary material Fig. S6E). Under conditions favoring lineage specification and differentiation, at 96 h ~30% of cells were Olig2\(^{+}\), and this was unchanged by ActB or Tgf\(\beta\)1 treatment (supplementary material Fig. S6F).

Together, these results suggested that Tgf\(\beta\)1 and ActB promote neural progenitor viability, compatible with outcomes in specified OLPS, but we detected no impact on proliferation or oligodendrocyte lineage specification.

DISCUSSION

Smad3 signaling supports myelination in the developing spinal cord

In this study, we show that combinatorial signaling by Tgf\(\beta\)1 and Activin ligands supports oligodendrocyte development and CNS myelination. Using mouse spinal cord as a model system, we found that at E12.5, following ventral OLP specification, canonical Smad3 activity localizes to the Sox2\(^{+}\) periventricular zone, including Olig2\(^{+}\) OLPS in the pMN domain. Our data suggest that Smad3 activity in the oligodendrocyte lineage is likely to result initially from exposure to Tgf\(\beta\)1-3 in the periventricular neuroepithelium, then later from combined exposure to Tgf\(\beta\)1 plus ActB in the parenchyma, particularly during differentiation in late embryogenesis and the early postnatal
We show that Tgfβ1 and ActB differentially activate canonical Smad3 and non-canonical Smad-independent MAP kinase signaling in OLPs in vitro, and that co-treatment produces a third response, strongly activating both signaling pathways. Moreover, each pattern correlates with a distinct functional outcome. Each ligand inhibits apoptosis, and Tgfβ promotes proliferation whereas ActB enhances maturation. In combination, they further enhance viability, and their effects on proliferation and maturation are combined, resulting in a potent increase in mature oligodendrocytes. Co-treatment promotes myelination in OLP-neuron co-cultures, and maturing oligodendrocytes in myelinating spinal cord are strongly positive for Smad3 and MAP kinase activation. In mice, inactivation of ActB
alone results in a transient reduction in Olig2+ cells and increased apoptosis, but numbers, differentiation and myelination rapidly recover. Importantly, in Smad3−/− mice, the numbers of mature and immature Olig2+ cells are more persistently reduced, indices of viability and proliferation are diminished, and myelination is delayed.

Smad3 activity in Olig2+ OLPS in periventricular neuroepithelium of mouse spinal cord

Our expression data are compatible with and extend previous studies. ActrIIb has been reported in mouse and chick neural tube at time points including those of OLP specification (Feijen et al., 1994; Stern et al., 1995), and the data from mouse match our own findings. Inhbb
mRNA has been detected in mouse neural tube at similar time points (Feijen et al., 1994), although the longitudinal sections presented render comparison with our own results difficult. Interestingly, in situ hybridization studies in chick have also reported Smad3 mRNA within periventricular neuroepithelium, although the signal in the pMN domain was relatively low at HH stages 25-26 (E4.5-5.0, equivalent to mouse E13), and absent at stages 12-18 (E2.0-3.0, equivalent to mouse E10-11.5) (Garcia-Campmany and Marti, 2007; Estaras et al., 2012). In the chick, OLPs emerge from ventral neuroepithelium as in rodents, beginning at E6.0 (HH stages 28-29) (Ono et al., 1995). In our studies in mouse, the double-labeling data confirm Smad3 expression and activity (P-Ser423/425) at E12.5 in OLPs in the pMN domain, compatible with the pattern throughout the periventricular neuroepithelium, and these findings also match our data for Tgfβr2 and Tgfβ1-3, which are compatible with previous reports (Flanders et al., 1991; Mecha et al., 2008). Thus, in mouse, the ligands, receptor and active form of the transcription factor are present at E12.5 throughout the periventricular neuroepithelium, including Olig2+ cells in the pMN domain. Whether a slight species difference exists in the timing of Smad3 expression and oligodendrocyte generation is not yet clear, and comparative studies will be needed to resolve this question.

**Transition of Tgfβ and Activin signaling in vivo – from distinct to overlapping patterns**

We tracked longitudinal changes in expression within spinal cord and, importantly, these analyses revealed that during embryogenesis the patterns of Tgfβ1 versus ActB switch from differential to overlapping, and are reflected in changes in Smad3 and MAP kinase activity, although an important qualifier is that MAP kinase pathways are also activated by factors beyond the Tgfβ superfamily (Bhat and Zhang, 1996; Althaus et al., 1997; Yim et al., 2001). Our mechanistic experiments demonstrate functional responses to these ligands individually and in combination, and our data from *Inhbb*–/– and *Smad3*–/– mice are compatible with these outcomes. That the *Smad3*–/– phenotype is the more severe might be attributable to complete versus partial ablation of Tgfβ1/Activin Smad3-dependent signaling. The compensation seen in both genotypes also follows a precedent in other models displaying deficits in oligodendrocyte development and/or myelination (Montag et al., 1994; Larsen et al., 2006; Lewallen et al., 2011), although recovery is not a universal outcome (Meyerheim et al., 2004; Nolan et al., 2005; Emery et al., 2009). Our findings are also compatible with data from previous studies in mutants for MAP kinase components (Schwammenthal et al., 2004; Fyffe-Maricich et al., 2011).

Notably, our data do not exclude potential contributions from other ligand-receptor combinations known to induce Smad3 activation, and such contributions would not discount the significance of our findings. Our data demonstrate that combinatorial effects of Smad3-activating ligands enhance oligodendrocyte development. Moreover, and importantly, we show that the net impact of Smad3 signaling in vivo (from all potential sources) promotes myelination in mammalian spinal cord.

**Roles of Tgfβ and Activin ligands in oligodendrocyte development**

The impact of ActB and Tgfβ1 on OLP viability and differentiation in our *in vitro* experiments is compatible with the findings of previous studies in CNS neuronal cultures (Schubert et al., 1990; Poulsen et al., 1994; Krieglstein et al., 1995; Cambray et al., 2012). Reports also suggest potential roles for Tgfβ and Activin ligands in repair in demyelinating models (Hinks and Franklin, 1999; John et al., 2002; Miron et al., 2013). Interestingly, however, our data do not match a report in Oli-Neu cells that implicated ActA in the causation of apoptosis (Schulz et al., 2008). Although ActA is not found in the developing CNS (Feijen et al., 1994), in our own experiments in primary OLPs we have found that it produces a close match to the effects of ActB, and so the source of this dichotomy is not immediately clear. Our findings showing that Tgfβ1 supports OLP proliferation are compatible with results in Schwann cells, in which it also acts as a mitogen (Ridley et al., 1989; Einheber et al., 1995). However, in studies in aggregate CNS cultures, addition of Tgfβ1 has been shown to enhance myelination (Diemel et al., 2003), whereas Tgfβ1 inhibition potentiates platelet-derived growth factor-aa (Pdgf-aa)-driven proliferation in O-2A cells (McKinnon et al., 1993). Notably, aggregate cultures are known to contain Tgfβ-producing cells, and, based on our data, ActB would also likely be present. Based on our results, addition of Tgfβ1 in the presence of ActB might be expected to lead to an overall increase in mature oligodendrocytes; thus, the two studies might be compatible. While the extent to which O-2A cells are representative of specified OLPs is not clear, we carefully examined effects on proliferation in our experiments in *Smad3*–/– mice. Since Pdgfγr signaling is an important mitogen for OLPs (Raff et al., 1988), we examined whether *Smad3* inactivation in vivo would result in detectable changes in OLP proliferation and, if so, whether a decrease or increase would result. However, we detected significant reductions in the fraction of Ki67+ Olig2+ cells in *Smad3*–/– samples at both E12.5 and posteriorly, compatible with our results in OLP cultures.

**Tgfβ and Activin signaling in white matter formation**

Taken together, our findings suggest a model of how Smad3-activating ligands might contribute to oligodendrocyte development in mammalian spinal cord. Initially, Tgfβ ligand-activated canonical and non-canonical signaling might support viability and proliferation in specified OLPs. As these cells spread dorsally within the cord, they remain under the influence of Tgfβ ligands, and also encounter ActB within the parenchyma. The combination of Tgfβ and Activin signaling further promotes viability, and effects on proliferation and maturation are combined, resulting in increased numbers of mature cells available for myelination.

**MATERIALS AND METHODS**

**Antibodies**

Phospho-Smad2 (Ser465/467), Smad2, phospho-Smad1/5/8 (Ser463/465), Smad3, phospho-Smad3, cleaved caspase-3, phospho-Gsk3β (Ser21/9), p38 (Mapk14), phospho-p38 (Thr180/Tyr182), phospho-p44/42 (Mapk3/1) (Thr202/Tyr204) and Foxh1 (FAST) were from Cell Signaling. Tgfβr2, Tgfβ1, Tgfβ2, Tgfβ3, Pdgf γr, β-actin, Bmpr2, rabbit IgG and mouse IgG were from Santa Cruz Biotechnology. Cnpase, Olig2, Ki67, Lim1 (Lhx1), Sox9, nestin, Sox2, NeuN (Rbfox3) were from Millipore. Other antibodies: Mbp were from Dako; Mbp, mouse CC-1 (Apc) and Gfap were from Acris; Mag was from Dr Jim Salzer (NYU); BrdU was from Immunology Consultants; ActrIIa (Acvr2a) and ActrIIb (Acvr2b) were from Abbiotec; Isl2 and Mnr2 (Mnx1) were from DSHB (University of Iowa); Inhba and Inhbb were from Proteintech. Please see supplementary material Table S1 for a complete list of antibodies used, species, dilutions and catalog numbers.

**Cytokines/growth factors**

Human ACTB, TGFβ1 and BMP4 were from Peprotech and used at 1-100 ng/ml. EGF was from Invitrogen and used at 20 ng/ml.

**OLP cultures**

OLPs were purified from cortices of P2 Sprague Dawley rats as reported (Caparoso and Chao, 2001). At plating, these cultures are >95% Olig2+ A2B5+ Pdgfγr+ Cnpase+ Mbp− OLPs (Gurfein et al., 2009). Cells were
plated in minimal essential medium (MEM) plus 15% FBS (Gibco), then changed to serum-free Bottenstein-Sato medium (Gibco) and continuously exposed to 1-100 ng/ml Tgfβ1, ActB and/or Bmp4 for up to 5 days.

Myelinating co-cultures
DRG neurons were isolated from E16.5 rat embryos as reported (Chan et al., 2004). Rat OLs (as above) were then plated onto DRG neurons and myelination induced using 1 µg/ml TrkA-Fc (Chan et al., 2004). Co-cultures were fixed at 14 days to assess myelination.

Adherent neural progenitor cultures
The protocol and proprietary reagents from STEMCELL Technologies were used to generate monolayer neural progenitor cultures (Conti et al., 2005; Louis et al., 2013). Ganglionic eminences from E14 mouse embryos were dissected and triturated, and dissociated cells plated into proprietary proliferation media containing 20 ng/ml recombinant human EGF (Invitrogen). To induce differentiation, cells were plated into proprietary growth-factor-free media for up to 96 h.

Bromodeoxyuridine (BrdU) incorporation
10 µM BrdU (BD Biosciences) was added to cells 12 h prior to fixation. Cells were stained for BrdU as per the manufacturer’s instructions.

Immunoblotting and co-immunoprecipitation
SDS-PAGE and immunoblotting were carried out as described (Zhang et al., 2011). Co-immunoprecipitation was performed for Smad3 or using IgG control was performed in Oli-Neu or HEK cell cultures using previously reported protocols (Attisano et al., 2001).

Immunocytochemistry
OLP or neural progenitor cultures, and OLP-neuron co-cultures, were fixed for 10 min with 4% paraformaldehyde and immunostained as previously described (Zhang et al., 2006, 2009). For surface antigens, PBS was used as buffer. For intracellular antigens, PBS containing 0.3% Triton X-100 was used. 10 µM BrdU (BD Biosciences) was added to cells 12 h prior to fixation. Cells were stained for BrdU as per the manufacturer’s instructions.

Mice
Inhbb−/− mice were made by Dr Rudolf Jaenisch (MIT) and display failure of eyelid fusion and impaired female reproduction (Vassalli et al., 1994). Smad3−/− (Smad3−/−) mice were made by Dr Chuxia Deng (NIH) and are immunodeficient and die before 8 months of age (Yang et al., 1999). C57BL/6 mice were from Taconic. Experiments involving animals were approved by the Institutional Animal Care and Use Committee, and conform to institutional and national animal welfare laws, guidelines and policies.

Immunohistochemistry
Frozen sections were rehydrated in PBS, then subjected to epitope retrieval in citrate buffer (pH 6) at 95-100°C for 10-15 min and immunostained using published protocols (Zhang et al., 2006, 2009).

Morphometric analysis
Differentiation, proliferation and apoptosis in OLP or neural progenitor cultures was quantified in z-stacks at 20×. Positive cells were counted in five 20× fields of vision per condition by a blinded observer and data compared statistically. In OLP-neuron co-cultures, myelin segments were identified as Mbp+ linear profiles extending along Nf+ axons. Number and length were quantified by a blinded observer in five stacks per condition. To characterize expression patterns in wild-type mouse embryos and the phenotypes of Inhbb−/− and Smad3−/− mice, cell number, myelinated area, or mean pixel intensity in cells marked with lineage or maturation markers were carried out in at least three fields in images of immunolabeled sections at 20× magnification per animal, three animals per genotype or condition per time point, in at least three independent experiments per study by a blinded observer.

Modeling
A previously described model (Zhang et al., 2011; Hsieh et al., 2004) was adapted to separate effects of ActB on OPC proliferation, apoptosis and differentiation (Fig. 4). The model calculates the proliferation coefficients of the Olig2 Mbp+ (OLP plus immature) (α) and mature Olig2 Mbp+ oligodendrocyte populations (γ), differentiation from one to the other (β), and the cell cycle coefficient of apoptosis of OLps plus mature cells δ+ε (Fig. 4A). These take into account the rate of change of the measured parameters and the size of the population (Hsieh et al., 2004).

The model uses differential equations to generate coefficients α through ε:
\[
dN_{OLP}/dt = (\alpha - \delta)N_{OLP} - \beta N_{OLP},
\]
the change in OLP percentage (N_{OLP}) over time.

\[
dN_{MBP}/dt = (\gamma - \epsilon)N_{MBP} + \beta N_{OLP},
\]
the change in mature cell percentage (N_{MBP}) over time.

\[
dN_{Total}/dt = (\alpha - \delta)N_{OLP} + (\gamma - \epsilon)N_{MBP},
\]
the change in total cell number over time.

\[
BrdU_{i+1} = 2\gamma N_{OLP} + 2\alpha N_{OLP},
\]
mitotic cells (BrdU+) between times i and i+1.

\[
BrdU_{i+1} = 2\gamma N_{OLP} + 2\alpha N_{OLP},
\]
mitotic cells (BrdU+) between times i and i+1.

\[
BrdU_{i+1} = 2\gamma N_{OLP} + 2\alpha N_{OLP},
\]
mitotic cells (BrdU+) between times i and i+1.

\[
BrdU_{i+1} = 2\gamma N_{OLP} + 2\alpha N_{OLP},
\]
mitotic cells (BrdU+) between times i and i+1.

\[
BrdU_{i+1} = 2\gamma N_{OLP} + 2\alpha N_{OLP},
\]
mitotic cells (BrdU+) between times i and i+1.

\[
BrdU_{i+1} = 2\gamma N_{OLP} + 2\alpha N_{OLP},
\]
mitotic cells (BrdU+) between times i and i+1.

\[
BrdU_{i+1} = 2\gamma N_{OLP} + 2\alpha N_{OLP},
\]
mitotic cells (BrdU+) between times i and i+1.


