Regeneration of axons in injured spinal cord by activation of bone morphogenetic protein/Smad1 signaling pathway in adult neurons

Pranav Parikh^{a,b,1}, Yuhan Hao^{a,b,1}, Mohsen Hosseinkhani^{a,b}, Shekhar B. Patil^a, George W. Huntley^a, Marc Tessier-Lavigne^c, and Hongyan Zou^{a,b,2}

^aFishberg Department of Neuroscience and ^bDepartment of Neurosurgery, Friedman Brain Institute, Mount Sinai School of Medicine, New York, NY 10029; and ^cGenentech, Inc., South San Francisco, CA 94080

Edited by Mark H. Tuszynski, University of California at San Diego, La Jolla, CA, and accepted by the Editorial Board April 1, 2011 (received for review January 11, 2011)

Axon growth potential is highest in young neurons but diminishes with age, thus becoming a significant obstacle to axonal regeneration after injury in maturity. The mechanism for the decline is incompletely understood, and no effective clinical treatment is available to rekindle innate growth capability. Here, we show that Smad1-dependent bone morphogenetic protein (BMP) signaling is developmentally regulated and governs axonal growth in dorsal root ganglion (DRG) neurons. Down-regulation of the pathway contributes to the age-related decline of the axon growth potential. Reactivating Smad1 selectively in adult DRG neurons results in sensory axon regeneration in a mouse model of spinal cord injury (SCI). Smad1 signaling can be effectively manipulated by an adeno-associated virus (AAV) vector encoding BMP4 delivered by a clinically applicable and minimally invasive technique, an approach devoid of unwanted abnormalities in mechanosensation or pain perception. Importantly, transected axons are able to regenerate even when the AAV treatment is delivered after SCI, thus mimicking a clinically relevant scenario. Together, our results identify a therapeutic target to promote axonal regeneration after SCI.

intrinsic axon growth capacity | intrathecal viral vector delivery

Spinal cord injury (SCI) disrupts long-projection axons, with devastating neurological outcomes, yet no effective clinical treatment exists. Neurons fail to regenerate axons because of a growth-inhibiting environment at the injury site (1–4) and because of an age-dependent decline in the intrinsic axon growth potential (5, 6). Nevertheless, blocking extracellular inhibitory molecules (7–10) or alleviating the intracellular negative regulators of axonal growth (5, 6, 11, 12) enables only limited axonal regeneration. Thus, additional molecular pathways that can rekindle innate growth capability must exist but remain unidentified (13).

Dorsal root ganglion (DRG) neurons are a favored model system to study axonal regeneration. These neurons have an axon with two branches—a peripheral branch that innervates sensory organs and a central branch that relays information to the CNS. The central branches of adult DRG neurons in the spinal cord are refractory to regeneration unless their peripheral branches are severed first. This so-called “conditioning lesion” paradigm activates a transcription program that enhances the intrinsic axonal growth potential (14). Previously, through gene expression profiling, we have demonstrated that Smad1 is induced after peripheral axotomy and that intraganglionic delivery of bone morphogenetic protein 2 or 4 (BMP2 or -4) activates Smad1 and enhances the axon growth potential of adult DRG neurons in cultures. In contrast, severing the central branches of DRGs fails to activate the Smad1 pathway, which correlates with the absence of regeneration after SCI (15).

These results suggested a possible involvement of Smad1 in regulating the growth state of DRG neurons. It is not known, however, whether Smad1 governs the axon growth program in young neurons and whether down-regulation of this pathway underlies the age-related decline of the intrinsic axon growth potential. Furthermore, it remains to be determined whether failure

to reactivate Smad1 contributes to a lack-of-growth state after SCI and whether empowering older neurons with increased Smad1 signaling can promote axon regeneration after SCI in vivo. Here we show that Smad1-dependent BMP signaling is developmentally regulated and governs axon growth potential and that activating Smad1 in adult DRG neurons by adeno-associated virus (AAV)-BMP enhances regrowth of adult sensory axons in vivo.

Results

Smad1 Is Developmentally Regulated in DRG Neurons and Governs Axon Growth Potential. Smads are the intracellular mediators of the TGF- β /BMP signaling pathway. TGF- β /BMP ligands activate receptor serine/threonine kinases, which in turn signal through C-terminal phosphorylation of Smads, leading to nuclear translocation of pSmads. Smad1, -5, and -8 mediate signaling of members of the BMP subfamily (16). We first examined whether Smad1 is expressed in embryonic DRG neurons during the period of active axon growth. In situ hybridization of embryonic spinal cord showed that *Smad1* was strongly expressed in the embryonic day (E)12.5 spinal cord and DRGs (Fig. 1A). In contrast, *Smad5* transcripts were mostly detected in the periventricular zone of the developing spinal cord, whereas *Smad8* was expressed at low level in E12.5 DRGs (Fig. S1A). Thus, Smad1 seems to be the dominant Smad that mediates BMP signaling in developing DRG neurons. We also examined Smad2, a mediator of the TGF- β subfamily, and found less-abundant expression level in E12.5 DRGs (Fig. S1A).

We next conducted a time course analysis. *Smad1* started to be expressed in DRGs at E10.5 and persisted through E15.5 (Fig. S1B). Consistent with its role as a transcription factor, we found abundant phosphorylated Smad1 (pSmad1) in the nuclei of embryonic DRG neurons by immunohistochemistry (Fig. 1E and Fig. S1C). In contrast, in adult DRG neurons, pSmad1 is down-regulated (Fig. 1F) but reappears after a conditioning lesion (Fig. 1G) (15). The dynamic expression pattern of pSmad1 coincides with the changes of the intrinsic axon growth potential: embryonic and conditioned adult DRG neurons were able to extend much longer axons than adult naïve neurons in dissociated cultures (Fig. 1B–D and H). pSmad1 was also found in the nuclei of motor neurons in the developing spinal cord and facial nucleus, as well as in Purkinje, retinal ganglionic, and olfactory

Author contributions: H.Z. designed research; P.P., Y.H., M.H., S.B.P., and H.Z. performed research; S.B.P., G.W.H., and M.T.-L. contributed new reagents/analytic tools; P.P., Y.H., M.H., G.W.H., and H.Z. analyzed data; and G.W.H., M.T.-L., and H.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. M.H.T. is a guest editor invited by the Editorial Board.

¹P.P. and Y.H. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: hongyan.zou@mountsinai.org.

See Author Summary on page 7661.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1100426108/-DCSupplemental.

be cell autonomous: neurons in low-density dissociated cultures showed a similar dose-dependent inhibition (Figs. 1 *K* and *L*). When BMP signaling was blocked, growth-associated protein 43 (GAP-43), a marker for active axon growth, was significantly decreased (Fig. S2*F*), and growth cones appeared dystrophic (Fig. 1*K*). BMP signaling is not only essential for axonogenesis of peripheral nervous system (PNS) neurons, it also seems to be indispensable for neurite outgrowth of CNS neurons. Embryonic day 18.5 hippocampal neurons could not initiate or maintain neurite outgrowth in explant cultures in the absence of BMP signaling (Fig. S2 *G–M*). Neuronal survival was not affected, because axons resumed growth after DM washout (Fig. S2*N*). To further confirm that it is the canonical Smad1-dependent BMP pathway that is critical for axon growth, we knocked down *Smad1* by RNAi in E12.5 DRG neurons and found that the axonal growth capacity was severely inhibited, an effect that could be rescued by an RNAi-resistant Smad1 construct (Fig. 1 *M* and *N*). In contrast, stimulation by exogenous BMP led to a further increase in the nuclear accumulation of pSmad1 and concurrent enhancement of axon growth potential (Fig. 1 *O–Q*).

BMP/Smad1 Signaling Is Essential for the Conditioning Effect in Adult DRG Neurons. We next asked whether the conditioning effect in adult DRG neurons is based on reactivation of Smad1. Dissociated adult DRG neurons start to extend axons after a 1-d delay, representing an in vitro conditioning process, because the dissociation step severs peripheral axons. When BMP signaling was blocked by DM treatment immediately after plating, the initiation of axonal outgrowth of the dissociated DRG neurons was significantly inhibited, whereas neuronal survival was not affected because axons resumed growth after DM washout (Fig. 2 *A* and *B*). In support of a Smad1-dependent BMP signaling cascade that governs axon outgrowth, a time course study revealed a lag time of ≈ 9 h between the addition of DM and the arrest of axon outgrowth (Fig. 2*C*). This lag time suggests that BMP signaling operates through a change on the transcriptional level, whereas an effect through LIM kinase-mediated local cytoskeletal stability seems less likely (18, 19). Indeed, DM did not cause an acute collapse of growth cones; rather, a depletion of pSmad1 in nuclei was observed (Fig. 2*D*). A delayed response to DM inhibition was also observed in dissociated DRG neurons that were conditioned in vivo by sciatic nerve transection. These in vivo conditioned DRG neurons were able to initiate but not maintain axon growth in cultures (Fig. 2 *E* and *F*), suggesting that the downstream effectors controlling the axon growth capacity were already operational at the beginning owing to the conditioning lesion but required a sustained BMP signaling cascade to maintain their expression. Smad1 knockout mice are early-embryonically lethal because of defects in allantois development (20); we thus bred mutant mice with *Smad1* loxP alleles (21) to the *Wnt1*-Cre line (22) to generate *Smad1* conditional knockout (CKO) mice. DRG neurons from *Smad1*^{lox/+}; *Wnt1* Cre mice had no detectable Smad1 (Fig. 2*I*); by comparison, abundant Smad1 was seen in DRG neurons from *Smad1*^{lox/+}; *Wnt1* Cre control littermates (Fig. 2*I*). When DRG neurons from the *Smad1* CKO mice were cultured, they displayed markedly decreased capability to initiate or maintain axonal extension (Fig. 2 *G* and *H*). These results support a model in which reactivation of the Smad1-dependent BMP pathway is critical for rekindling the innate growth potential in adult sensory neurons.

In Vivo Activation of Smad1 Enhances the Axon Growth Potential of Adult DRG Neurons. We then set out to test whether in vivo activation of Smad1 in adult DRG neurons could promote sensory axon regeneration in a rodent SCI model. We designed an AAV-based strategy, coupled with a clinically applicable and minimally invasive delivery method. We reasoned that delivering AAV directly into the lumbar cerebrospinal fluid space would lead to widespread distribution of the viruses to target multilevel DRGs. DRGs reside at the end of nerve root sleeves surrounded by cerebrospinal fluid—providing the basis for intrathecal delivery

(Fig. 3*A*). Intrathecal AAV-GFP injection led to high and selective transduction of DRG neurons (up to 50% in lumbosacral, 12% in thoracic, and 30% in cervical DRGs) (Fig. 3*B* and Fig. S3 *A* and *B*). GFP was well visualized within individual axon fibers in both the peripheral and the central branches of DRG (Fig. 3*B* and *C*). We observed no GFP⁺ glial cells in DRGs and only a few faintly GFP⁺ interneurons within the gray matter of the spinal cord, consistent with the reported tissue tropism of AAV (23). The rostral spread of the virus was limited to the cervical spinal cord, because GFP was not detected in the brain (Fig. S3*A*). GFP expression was first visible at day 7 and was sustained at 28 d after injection (Fig. S3*C*).

We first asked whether DRG neurons from the AAV-BMP4-injected mice have enhanced axon growth potential. We chose to test BMP4 in this study, because in our previous work intraganglionic injection of BMP4 enhanced axonal growth (15). The DRG neurons isolated from the AAV-BMP4-treated adult mice indeed extended much longer axons at 20 h than controls (Fig. 3 *D* and *E*). BMP4 expression was markedly increased, driven by AAV-mediated overexpression, and pSmad1 accumulated in the nuclei of DRG neurons in AAV-BMP4 mice (Fig. 3*F*). In agreement with BMP4 being a secreted ligand, we observed a “field effect” from overexpression of BMP4: in AAV-BMP4 mice the percentage of the lumbar DRG neurons that had nuclear pSmad1 was much higher than the AAV transduction rate. Remarkably, GAP-43 was induced in DRGs from AAV-BMP4 mice, as in conditioned DRGs. In contrast, ATF3, another regeneration-associated gene (24), was not induced (Fig. 3*F*). There were no detectable changes in the cellular components of the DRGs among treatment groups by histological analysis or cell type-specific markers (Fig. S3*D*).

Sensory Axon Regeneration by Activation of the BMP Pathway in a Mouse Model of SCI. Next, we tested AAV-BMP4 in a mouse SCI model of complete dorsal column transection (Fig. 4*A* and *B* and Fig. S4*A*). Mice were first injected with intrathecal AAV and 2 wk later received a T8 dorsal hemisection, thus allowing sufficient time for BMP4 overexpression to prime the DRG neurons. Regenerating axons were visualized by transganglionic labeling with Dextran-Texas Red (DexTR). In the control groups—sham, intrathecal saline, or AAV-GFP injection—at 2 wk after SCI, virtually all ascending fibers in the fasciculus gracilis had retracted from the caudal border of the lesion (Fig. 4 *C–E*, *H*, and *I*), consistent with the failure of regeneration in the CNS (25). In contrast, in the AAV-BMP4 group, regenerative responses were observed (Fig. 4 *F* and *J–M* and Fig. S5*E*). Significantly more injured fibers penetrated the caudal border of the lesion site, traversed the lesion epicenter, and even emerged from the rostral border (Fig. 4*G*). Most regenerating fibers tended to remain in bundles, ascending dorsally into the scar, and then renegotiated their passage back to deeper portions of the dorsal column. Thus, the regenerating axons have characteristic circuitous trajectories along the ventral–dorsal axis (Fig. 4 *L* and *M*, arrows), as opposed to the straight caudal-to-rostral trajectories of uninjured axons (Fig. 4 *H* and *I*). Another salient feature of the regenerating axons was the frequent dye-filled swellings at the axon tips, which resembled growth cone-like structures (Fig. 4 *L* and *M*, arrowheads, and Fig. S5). Lesion completeness was confirmed in every animal by the absence of tracer on cross-sections of rostral cervical spinal cord (Fig. S4). Sometimes microcysts developed in the lesion core, and large bundles of regenerating fibers grew along the cyst wall and extended further rostrally (Fig. 4*L*).

Confocal imaging revealed that regenerating axons tended to avoid high-density GFAP⁺ areas and chose to navigate the less dense GFAP⁺ areas (Fig. S5), in agreement with the model that reactive astrocytes secrete inhibitory extracellular molecules, such as chondroitin sulfate proteoglycans (CSPG) (26). The promoting effect most likely resulted from an enhanced intrinsic growth potential, because only DRG neurons were selectively targeted by AAV with our intrathecal delivery method. In fact, injection of AAV-GFP into the lumbar intrathecal space ei-

Fig. 3. Intrathecal AAV-GFP selectively transduces DRG neurons and AAV-BMP4 enhances axon growth potential. (A) Experimental scheme of intrathecal injection. (B) Two weeks after AAV-GFP injection, GFP was detected in DRG neurons and their axons in dorsal roots and sciatic nerve. Numerous GFP⁺ fibers in cauda equina highlighted the high transduction efficiency. (C) Longitudinal (Left) and cross-sections (Right) of thoracic spinal cord demonstrating that GFP labeled the ascending sensory axons in the dorsal column. Dashed white line denotes the midline. Tuj1 highlighted neuronal population (red). (D and E) DRG neurons from AAV-BMP4 mice extended much longer axons than those from control mice at 20 h in vitro in the neurite outgrowth assays. *** $P < 0.0001$. (F) Immunostaining showed that BMP4 expression was increased both in cytoplasm (arrows) and on cell surface (arrowheads), pSmad1 accumulated in nuclei, and GAP-43 induced in conditioned or AAV-BMP4 DRGs, compared with AAV-GFP and contralateral controls. ATF3 was not induced by AAV-BMP4. (Scale bars, 50 μm in F; 100 μm in B–D.)

Fig. 4. AAV-BMP4 promotes ascending sensory axon regeneration. (A and B) Experimental scheme. Step 1: intrathecal AAV injection. Step 2: T8 dorsal hemisection at week 2. Step 3: tracing of ascending sensory axons with DexTR at week 4. (C–F) Sagittal sections: rostral is to the left and caudal to the right. Lesion borders were defined by GFAP staining of the reactive astrocytes (blue) and marked with dashed white line, and asterisks denote the lesion center. In sham, saline, or AAV-GFP mice, DexTR⁺ axons had retracted from the caudal border of the lesion site. In AAV-BMP4 mice, bundles of DexTR⁺ axons had penetrated the lesion site, and some traversed the lesion epicenter and emerged from the rostral border. (G) There were significantly more axons closer to the lesion center in AAV-BMP4 mice than controls. Axon index is the ratio of the DexTR⁺ axon number at a specific location relative to the axon number at the most caudal point—100% at >0.4 mm. Data are shown as mean $\pm$ SEM. $^{**}P < 0.001$, $^{***}P < 0.0001$, two-way ANOVA with Bonferroni post hoc correction. (H–M) Sagittal images from another cohort of mice injected with AAV-GFP (H and I) or -BMP4 (J and K). (L and M) Magnification of J and K. Regenerating sensory axons traversed the lesion center (straight dashed line). Bundles of axons displayed circuitous trajectories (white arrows). Arrowheads point to the typical appearances of the tips of the regenerating axon. Curved dashed line depicts a microcyst at lesion center. (Scale bars, 100 μ m.)

Postinjury AAV-BMP4 Injection Promotes Sensory Axon Regeneration. We further asked whether a post-SCI injection of AAV-BMP4 would be sufficient to stimulate axonal regeneration, which represents a clinically relevant scenario. Animals received AAV-BMP4 injection 15 min after T8 SCI in the same anesthesia setting to avoid adverse effects from multiple sessions of anesthesia in a short period. Notably, we observed similar axonal

regrowth in these animals (Fig. 5 C–J). Therefore, AAV-BMP4 delivered shortly after SCI is sufficient to switch DRG neurons into an active growth state to overcome early stages of gliosis.

Discussion

Smad1-Dependent BMP Signaling Is Essential for Axonogenesis. BMPs play diverse roles in the developing nervous system, from early

Fig. 5. Intrathecal AAV injection has no adverse effects and sensory axons regenerate in mice with postinjury AAV injection. (A) Behavioral assessment of mechanical allodynia, which was detected in control mice with L5 sciatic nerve ligation but not in AAV or sham groups. y axis: von Frey hair threshold in grams on log scale. $*P < 0.05$, one-way ANOVA with Kruskal-Wallis test, followed by Dunn's multiple comparison test. (B) CD45 immunostaining did not reveal changes of the extent of infiltration of inflammatory cells in experimental vs. control groups. (C) Experimental scheme. AAV was injected 15 min after SCI, and DexTR was injected at week 4. (D–G) Sagittal sections of AAV-GFP (D and E) and -BMP4 mice (F and G). Asterisks denote lesion epicenter. Dashed lines delineate lesion borders, as visualized with GFAP staining (blue). (H and I) Magnification of F and G. Arrows: the most rostral front of regenerating fibers. Straight dashed line: the lesion center. (J) Axon index confirmed that significantly more fibers were closer to lesion center. (Scale bars, 50 μ m in B; 100 μ m in D–I.)

decision to form neural ectoderm to patterning and proliferation of the spinal cord (reviewed in ref. 29). Smad has also been implicated in mediating BMP signaling from axon terminals, through retrograde transport, to regulate spatial patterning of neurons

(30). Here we propose a role of the BMP/Smad1 pathway in mediating axonal growth during CNS and PNS development. pSmad1 accumulates in the nuclei of neurons during axonogenesis. The initiation and the elongation of neurite outgrowth of

both CNS and PNS neurons require BMP signaling. Exogenous BMP stimulation enhances axon growth potential. As neurons mature, the axon growth capability is diminished, coinciding with the decrease of nuclear pSmad1 in older neurons. A conditioning lesion induces and activates Smad1 in adult DRG neurons and increases the axon growth potential. Therefore, a conditioning lesion recapitulates, at least in part, the developing process of axon growth. Indeed, blocking BMP signaling in adult DRG neurons by pharmacological inhibition, genetic ablation of Smad1, or acute Smad1 knockdown by RNAi all lead to failure of initiation of axonal outgrowth or arrest of axonal elongation, whereas reactivating Smad1 signaling in adult DRG neurons *in vivo* by AAV-BMP4 results in induction of regeneration markers and rekindling of axon growth potential.

A large number of growth factors and guidance molecules, such as neurotrophins, netrin, and Wnts, are able to enhance axonal outgrowth (31). Our results now add another classic signaling pathway to the growing family of growth factors that can mediate axon growth. Interestingly, Smad1 can act as a converging node integrating various signaling pathways through its linker phosphorylation at multiple MAPK and GSK3 phosphorylation sites. For example, in *Xenopus* embryos, dorsoventral (BMP) and anteroposterior (Wnt/GSK3) patterning gradients are integrated at the Smad1 linker area (32). By analogy, BMP and NGF signaling pathways may also converge on Smad1 through differential phosphorylation to mediate axon growth.

Although we have focused here on the role of Smad1-dependent BMP signaling in axon growth, our results do not exclude functions of BMPs at the tips of axons, independent of nuclear signaling. Indeed, noncanonical BMP signaling pathways have been implicated in mediating local effects of BMPs—regulating actin dynamics in dendritogenesis (19), acute growth cone collapse (33), and synaptic stability (18).

Activation of Smad1 Promotes Axonal Regeneration in SCI. Our studies show that empowering adult neurons by increasing BMP signaling *in vivo* can enhance axon growth potential, thereby promoting axon regeneration in a mouse model of SCI. The phenotype could in part be caused by a lack of axonal dieback of neurons with AAV-BMP4 treatment, as has been shown in conditioned adult neurons (34, 35). However, there seems to be genuine axonal regrowth: some axons did extend further rostrally, beyond the transection site, and even emerged from the rostral border of the lesion site. In addition, we observed a similar axonal regrowth phenotype when AAV-BMP4 was injected after SCI, in which case sufficient BMP4 was expressed after the acute axon dieback had occurred. Thus, besides potentially preventing acute axonal dieback, AAV-BMP4 mostly likely can also counteract the typical abortive attempt of axonal regeneration. The regeneration phenotype of AAV-BMP4 seems to be comparable to and, in some cases, even slightly more robust than the conditioning lesion in our mouse model of SCI, implying that AAV-BMP4 may not be simply a recapitulation of a conditioning lesion and that BMP4 may have recruited other signaling molecules beyond those activated by the conditioning lesion. In fact, BMP4 expression level with AAV-BMP was much higher than in conditioned DRGs (Fig. 3F), thus neurons may be stimulated by BMP4 with greater magnitude and longer duration. In addition, as a secreted ligand, BMP4 may affect not only transduced DRG neurons but also neighboring nontransduced neurons through a paracrine fashion.

Although previous studies have shown that manipulation of the BMP pathway locally at the injury site affects astrogliosis (36) and may inhibit regeneration (37), our studies activated the BMP/Smad1 pathway directly in sensory neurons within DRG, thus demonstrating that enhancing neuronal intrinsic axon growth potential can be used as a strategy to promote axon regeneration *in vivo*. Our AAV-based *in vivo* gene delivery method selectively targeting DRG neurons is minimally invasive, clinically applicable, and represents a versatile experimental manipulation for SCI research. This is in contrast to other manipulations, such as intraganglionic injection of cAMP (38, 39) or an inflammatory agent,

zymosan (27, 28), both of which are not clinically feasible. Our AAV intrathecal injection is also an approach devoid of unwanted abnormalities in mechanosensation or pain perception, thus providing the basis for future translation into clinical treatment.

AAV-BMP4 injected immediately after the SCI still leads to sensory axon regeneration. Reportedly, glial and fibrous scars begin to form 1 wk after injury and further mature until 3 wk after injury (40). This is consistent with prior observations that animals that received a peripheral axotomy concomitant with a central lesion show some degree of regeneration, whereas animal that received peripheral axotomy 2 wk after the SCI show no regeneration (41), implying that the first 2 wk might be a window of opportunity for treatment. We based our time estimate of 7 d for the onset of transgene expression on the observation that GFP becomes visible at day 7; however, because we did not use GFP antibody to enhance detection sensitivity, transgene expression might have earlier onset. Apparently, the low level of BMP4 in the first few days after AAV injection is sufficient to switch DRG neurons into an active growth state. Because the initiation of the AAV transgene expression is on the order of days, not hours, we predict that AAV-BMP delivered a few hours or 1 d after the SCI—a more practical regimen for treating acute SCI—should have similar promoting effects as when AAV-BMP4 was injected 15 min after SCI. Future studies will explore whether AAV-BMP is effective for subacute or even chronic SCI. A self-complementary recombinant AAV8 (sc-AAV8, engineered to be double stranded) with faster onset of transgene expression (23) might be crucial to rejuvenate neurons before astroglial scars become an impenetrable barrier for regeneration. Combinatorial strategies, such as relieving inhibition from CSPGs (42), cell grafting, or placement of growth factor gradients or guidance cues beyond lesion epicenter (43, 44), might lead to synergistic effects. In fact, a time course study at 4 wk after SCI showed no further regeneration beyond the 2 wk after lesion, highlighting the importance of combinatorial strategies.

Future studies will also determine the therapeutic potential of AAVs encoding other subtypes of BMPs (in particular, BMP7, which has an enhancing effect on embryonic neurons, as shown in Fig. 1O) or other components of the BMP signaling pathway for sensory axon regeneration. In addition, BMP signaling for the regeneration of motor fibers in the corticospinal tract (CST) can be readily tested: our pilot experiment demonstrated a similar decline of the pSmad1 in mature cortical motor neurons. The role of the Smad1/BMP4 pathway in the normal axonal regeneration of peripheral nerve after injury also awaits future studies.

Smad1 has also been found to be increased in cortical neurons that sprout a new connection after stroke as part of the neuronal growth program identified as “sprouting transcriptome” (45). Additionally, BMP7 infusion has neuroregenerative effects after stroke (46), suggesting that the promoting effect of BMP/Smad1 signaling is not limited to SCI.

Fig. 6. Working model. Age-dependent decline of the axon growth potential and the concurrent down-regulation of Smad1. A peripheral lesion rekindles the innate growth potential, whereas a central lesion in SCI does not lead to regeneration. Reactivating Smad1 either before or after SCI enhances the growth potential, thereby promoting axonal regeneration. y axis: relative neuronal regeneration capacity.

Taken together, we have found an essential role of the BMP/Smad1 pathway in axonogenesis during development and in rekindling the innate growth potential in adult sensory neurons (Fig. 6). Importantly, we discovered a promoting effect of AAV-BMP4 in the regeneration of long-projection sensory fibers in a rodent model of SCI. Modulating the BMP/Smad1 pathway thus represents a therapeutic strategy for axonal regeneration.

Materials and Methods

Mice, Conditioning Lesion, and Thoracic Dorsal Hemisection. All surgeries were performed on adult female mice 4–6 wk old in accordance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee at the Mount Sinai School of Medicine. For the SCI model, C57BL/6 mice were used. Mice received ketamine and xylazine for anesthesia. Dorsal column transection injuries were performed as previously described (38, 41). Briefly, the lamina of T8 spinal segment was exposed and the dorsal columns transected bilaterally using iris microscissors (Fine Science Tools), with the depth reaching ≈ 0.8 mm. For the peripheral conditioning lesion, the right sciatic nerve was exposed at midhigh level and transected. *Smad1^{flox/flox}* mice and *Wnt1-Cre* line were obtained from Jackson Laboratories. We also used *Smad1^{flox/flox}* mice to generate heterozygous germ-line deletion of *Smad1* (*Smad1^{+/-}*).

AAV and Intrathecal Injection. For AAV preparation, cDNA of *BMP4* or *GFP* was inserted downstream of a CMV promoter in a recombinant AAV8.2 vector (Virovek). Viral titers were on the order of 1×10^{13} viral genomes per milliliter (vg/mL). Intrathecal injection was after the modified Wilcox technique (47). The site of injection was between lumbar levels L5 and L6, a location where the spinal cord ends and the cauda equina begins in mouse. Specifically,

a small laminectomy was performed to expose the thecal sac between L5 and L6. AAV particles on the order of 10^{10} vg in 3- μ L volume were injected using a 10- μ L Hamilton syringe with a 32-gauge needle. To avoid injury to the underlying neural tissue, the needle remained at midline and was slowly inserted underneath the dura and further advanced in the subarachnoid space. The technical parameters, such as isotonic diluent, low-infusion pressure, and a small injection volume, were all consistent with the clinical practice of intrathecal drug delivery. After the injection, the paraspinal muscles and fascia were reapproximated with 5-0 chromic sutures and skin with staples. Mice then received 0.5 mL normal saline and were returned to a warm chamber for postoperative recovery. For sham surgery, only laminectomy was performed, with no dural breachment. At least five mice were used for each experimental group. For AAV injection, experiments were repeated at least twice.

Labeling of Ascending Sensory Axons in the Fasciculus Gracilis. The ascending sensory fibers in the fasciculus gracilis was labeled unilaterally with Texas Red-conjugated Dextran 3000 MW (DexTR; Invitrogen) 3 d before perfusion. The right sciatic nerve was exposed and crushed with forceps. DexTR (2.5 μ L) was injected into the sciatic nerve using a Hamilton syringe at the crush site.

Statistical Analysis. Prism Graphpad software was used to perform Student *t* test, one-way ANOVA, or two-way ANOVA, followed by Bonferroni's post hoc test with multiple comparisons. Data are presented as mean $\pm$ SEM.

ACKNOWLEDGMENTS. We thank Karen Wong for technical support for in situ hybridization. This work was supported by the Whitehall Foundation, the Neurosurgery Research and Education Foundation of the American Association of Neurological Surgeons, and Mount Sinai Friedman Brain Institute (H.Z.).

- Filbin MT (2003) Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nat Rev Neurosci* 4:703–713.
- Liu K, et al. (2010) PTEN deletion enhances the regenerative ability of adult corticospinal neurons. *Nat Neurosci* 13:1075–1081.
- McGee AW, Strittmatter SM (2003) The Nogo-66 receptor: Focusing myelin inhibition of axon regeneration. *Trends Neurosci* 26:193–198.
- Schwab ME, Bartholdi D (1996) Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol Rev* 76:319–370.
- Moore DL, et al. (2009) KLF family members regulate intrinsic axon regeneration ability. *Science* 326:298–301.
- Park KK, et al. (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science* 322:963–966.
- Case LC, Tessier-Lavigne M (2005) Regeneration of the adult central nervous system. *Curr Biol* 15:R749–R753.
- Harel NY, Strittmatter SM (2006) Can regenerating axons recapitulate developmental guidance during recovery from spinal cord injury? *Nat Rev Neurosci* 7:603–616.
- Lee JK, et al. (2010) Assessing spinal axon regeneration and sprouting in Nogo-, MAG-, and OMgp-deficient mice. *Neuron* 66:663–670.
- Shen Y, et al. (2009) PTPsigma is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. *Science* 326:592–596.
- Smith PD, et al. (2009) SOCS3 deletion promotes optic nerve regeneration in vivo. *Neuron* 64:617–623.
- Sun F, He Z (2010) Neuronal intrinsic barriers for axon regeneration in the adult CNS. *Curr Opin Neurobiol* 20:510–518.
- Zhou FQ, Snider WD (2006) Intracellular control of developmental and regenerative axon growth. *Philos Trans R Soc Lond B Biol Sci* 361:1575–1592.
- Richardson PM, Issa VM (1984) Peripheral injury enhances central regeneration of primary sensory neurones. *Nature* 309:791–793.
- Zou H, Ho C, Wong K, Tessier-Lavigne M (2009) Axotomy-induced Smad1 activation promotes axonal growth in adult sensory neurons. *J Neurosci* 29:7116–7123.
- Massagué J, Seoane J, Wotton D (2005) Smad transcription factors. *Genes Dev* 19:2783–2810.
- Yu PB, et al. (2008) Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nat Chem Biol* 4:33–41.
- Eaton BA, Davis GW (2005) LIM Kinase1 controls synaptic stability downstream of the type II BMP receptor. *Neuron* 47:695–708.
- Lee-Hoeflich ST, et al. (2004) Activation of LIMK1 by binding to the BMP receptor, BMPRII, regulates BMP-dependent dendritogenesis. *EMBO J* 23:4792–4801.
- Lechleider RJ, et al. (2001) Targeted mutagenesis of Smad1 reveals an essential role in chorioallantoic fusion. *Dev Biol* 240:157–167.
- Huang S, et al. (2002) Conditional knockout of the Smad1 gene. *Genesis* 32:76–79.
- Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP (1998) Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr Biol* 8:1323–1326.
- Storek B, et al. (2008) Sensory neuron targeting by self-complementary AAV8 via lumbar puncture for chronic pain. *Proc Natl Acad Sci USA* 105:1055–1060.
- Seijffers R, Mills CD, Woolf CJ (2007) ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. *J Neurosci* 27:7911–7920.
- Ramon y Cajal S (1928) *Degeneration and Regeneration of the Nervous System* (Oxford Univ Press, New York).
- Silver J, Miller JH (2004) Regeneration beyond the glial scar. *Nat Rev Neurosci* 5:146–156.
- Steinmetz MP, et al. (2005) Chronic enhancement of the intrinsic growth capacity of sensory neurons combined with the degradation of inhibitory proteoglycans allows functional regeneration of sensory axons through the dorsal root entry zone in the mammalian spinal cord. *J Neurosci* 25:8066–8076.
- Yin Y, et al. (2003) Macrophage-derived factors stimulate optic nerve regeneration. *J Neurosci* 23:2284–2293.
- Liu A, Niswander LA (2005) Bone morphogenetic protein signalling and vertebrate nervous system development. *Nat Rev Neurosci* 6:945–954.
- Hodge LK, et al. (2007) Retrograde BMP signaling regulates trigeminal sensory neuron identities and the formation of precise face maps. *Neuron* 55:572–586.
- Rossi F, Gíanola S, Corvetto L (2007) Regulation of intrinsic neuronal properties for axon growth and regeneration. *Prog Neurobiol* 81:1–28.
- Fuentealba LC, et al. (2007) Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. *Cell* 131:980–993.
- Butler SJ, Dodd J (2003) A role for BMP heterodimers in roof plate-mediated repulsion of commissural axons. *Neuron* 38:389–401.
- Busch SA, Horn KP, Silver DJ, Silver J (2009) Overcoming macrophage-mediated axonal dieback following CNS injury. *J Neurosci* 29:9967–9976.
- Horn KP, Busch SA, Hawthorne AL, van Rooijen N, Silver J (2008) Another barrier to regeneration in the CNS: Activated macrophages induce extensive retraction of dystrophic axons through direct physical interactions. *J Neurosci* 28:9330–9341.
- Sahni V, et al. (2010) BMPRI1a and BMPRI1b signaling exert opposing effects on gliosis after spinal cord injury. *J Neurosci* 30:1839–1855.
- Matsuura I, Taniguchi J, Hata K, Saeki N, Yamashita T (2008) BMP inhibition enhances axonal growth and functional recovery after spinal cord injury. *J Neurochem* 105:1471–1479.
- Neumann S, Bradke F, Tessier-Lavigne M, Basbaum AI (2002) Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. *Neuron* 34:885–893.
- Qiu J, et al. (2002) Spinal axon regeneration induced by elevation of cyclic AMP. *Neuron* 34:895–903.
- Ylera B, et al. (2009) Chronically CNS-injured adult sensory neurons gain regenerative competence upon a lesion of their peripheral axon. *Curr Biol* 19:930–936.
- Neumann S, Woolf CJ (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. *Neuron* 23:83–91.
- Bradbury EJ, et al. (2002) Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* 416:636–640.
- Kadoya K, et al. (2009) Combined intrinsic and extrinsic neuronal mechanisms facilitate bridging axonal regeneration one year after spinal cord injury. *Neuron* 64:165–172.
- Alto LT, et al. (2009) Chemotropic guidance facilitates axonal regeneration and synapse formation after spinal cord injury. *Nat Neurosci* 12:1106–1113.
- Li S, et al. (2010) An age-related sprouting transcriptome provides molecular control of axonal sprouting after stroke. *Nat Neurosci* 13:1496–1504.
- Chou J, et al. (2006) Neuroregenerative effects of BMP7 after stroke in rats. *J Neurol Sci* 240:21–29.
- Hylden JL, Wilcox GL (1980) Intrathecal morphine in mice: a new technique. *Eur J Pharmacol* 67:313–316.