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A *Smad3* transgenic reporter reveals TGF-beta control of zebrafish spinal cord development

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ABSTRACT

TGF-beta (TGFβ) family mediated Smad signaling is involved in mesoderm and endoderm specifications, left–right asymmetry formation and neural tube development. The TGFβ1/2/3 and Activin/Nodal signal transduction cascades culminate with activation of SMAD2 and/or SMAD3 transcription factors and their overactivation are involved in different pathologies with an inflammatory and/or uncontrolled cell proliferation basis, such as cancer and fibrosis. We have developed a transgenic zebrafish reporter line responsive to Smad3 activity. Through chemical, genetic and molecular approaches we have seen that this transgenic line consistently reproduces *in vivo* Smad3-mediated TGFβ signaling. Reporter fluorescence is activated in phospho-Smad3 positive cells and is responsive to both Smad3 isoforms, Smad3a and 3b. Moreover, Alk4 and Alk5 inhibitors strongly repress the reporter activity. In the CNS, Smad3 reporter activity is particularly high in the *subpallium*, *tegumentum*, cerebellar plate, *medulla oblongata* and the retina proliferative zone. In the spinal cord, the reporter is activated at the ventricular zone, where neuronal progenitor cells are located. Colocalization methods show *in vivo* that TGFβ signaling is particularly active in *neuroD*+ precursors. Using neuronal transgenic lines, we observed that TGFβ chemical inhibition leads to a decrease of differentiating cells and an increase of proliferation. Similarly, *smad3a* and *3b* knock-down alter neural differentiation showing that both paralogues play a positive role in neural differentiation. EdU proliferation assay and pH3 staining confirmed that Smad3 is mainly active in post-mitotic, non-proliferating cells. In summary, we demonstrate that the Smad3 reporter line allows us to follow *in vivo* Smad3 transcriptional activity and that Smad3, by controlling neural differentiation, promotes the progenitor to precursor switch allowing neural progenitors to exit cell cycle and differentiate.

Introduction

TGFβ1/2/3, Activin/Nodal and BMP signaling belong to the TGFβ family. All three signaling subfamilies show a similar transduction pathway: the secreted ligands interact with type I and II transmembrane serine-threonine kinase receptors. The type II receptors phosphorylate type I receptor, which in turn permits the binding of receptor-regulated transcription factors SMADs (R-SMADs) and their subsequent phosphorylation at the C-terminus. Activated R-SMADs can interact directly with a common mediator, SMAD or Co-SMAD (SMAD4), and then translocate into the nucleus to directly target gene expression (Moustakas and Heldin, 2009).

BMP, Activin/Nodal and TGFβ1/2/3 signaling require different ligands, type II receptors (ALK1, 2, 3 and 6 for BMP; ALK4, 5 and 7 for TGFβ1/2/3 and Activin/Nodal) and R-SMADs (SMAD1, 5 and 8 for BMP; SMAD2 and 3 for TGFβ1/2/3 and Activin/Nodal) (Hinck, 2012). As a consequence, gene sequences recognized by R-Smads are different for the two signaling pathways. In zebrafish two *smad3* isoforms are known: *smad3a* and *3b*. They are the result of the genome duplication that occurred during teleost evolution. These two genes show a partially overlapping expression: they are both expressed in the tail bud and lateral stripes of the forming mesoderm; however, Smad3a is also produced in an additional area that surrounds the tail bud (Dick et al., 2000). Their mechanisms are similar and they are expressed in overlapping and non-overlapping tissues (Pogoda and Meyer, 2002) displaying additive genetic effects.

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All the TGFβ1/2/3 and Activin/Nodal *R-smads* and *smad4* transcripts are ubiquitously expressed since blastula stage as a consequence of their maternal origin (Dick et al., 2000). However, during gastrulation, they are either transcribed at very low level (*smad2*), or almost undetectable (*smad3a* and *3b*) (Dick et al., 2000; Pogoda and Meyer, 2002). In contrast, *smad4* expression is high in all these stages (Dick et al., 2000). From tail bud stage *smad3a* and *3b* mRNA production increases (Hsu et al., 2011). At late somitogenesis (16 hpf) *smad3a* mRNA is mainly confined to the eyes and tail region, although present at low levels throughout the embryo. *smad3b* is expressed in the same areas, but it has a higher rate of expression overall (Hsu et al., 2011). Both *smad2* and *4* are present in the entire embryo, but particularly in the tail region, the eye and the brain (Hsu et al., 2011). However, *smad2* and *3* expression is necessary but not sufficient for correct functionality: a signal transduction cascade leading to their phosphorylation is needed (Liu et al., 1997).

Although Smad2 and 3 share a highly similar protein structure (more than 90% amino acid sequence identity), they are involved in different physiological and pathological processes. Smad2 knockout mice fail to form mesoderm and endoderm demonstrating the importance of this transcription factor in early development (Nomura and Li, 1998). Smad3 knockout mice, while viable, have chronic intestinal inflammation producing colorectal cancer and metastasis (Zhu et al., 1998). Therefore, Smad3 transcription factor seems to be associated to the immune system and it might regulate cell cycle working as tumor suppressor. These different roles correlate with their slightly different structure. Smad2 contains an inhibitory region in the MH1 region that hinders direct DNA binding. In contrast, Smad3 recognize Smad Binding Element (SBE) boxes that were used for the creation of the Smad3-responsive line of this work. Furthermore, a Smad2 alternative splicing variant missing the inhibitory domain has been reported. This variant would bind DNA directly and is possibly responsible for Smad2's impact during development (Dunn et al., 2005; Lee et al., 2011).

R-SMADs activity is regulated by inhibitory-SMADs (I-SM ADs). For TGFβ, SMAD7 functions as a negative signaling regulator (ten Dijke and Hill, 2004). The inhibitory factor SMAD7 is induced by SMAD3 and provides a negative feedback loop to the pathway. In zebrafish, *smad7* shows a pattern of expression similar to that observed for *smad3b*, underlying the reciprocal functional connection between the two genes. *smad7* is ubiquitously present as maternal transcripts until gastrula stage, when its expression decreases, becoming limited to the ventral side of the embryo, though expression increases in the tail bud (Pogoda and Meyer, 2002). SMAD7 can act in different ways: it can compete with R-SMADs for binding type I receptors; it can recruit E3-ubiquitine ligases (SMURF1 and 2) to the activated type I receptors causing their degradation (ten Dijke and Hill, 2004).

TGFβ signaling is involved in a wide range of physiological and pathological processes in both embryonic and adult stages. It acts as a morphogen through Nodals and Activins directing the patterning of the three germ layers (Watabe and Miyazono, 2009). A dysregulation of this pathway is associated with tumorigenesis, fibrosis, allergic response and neurodegenerative diseases. Both in physiological and pathological conditions, its effect depends on the tight regulation of the cell cycle (Fleisch et al., 2006). TGFβ signaling is a well-known pro-apoptotic signal, it promotes epithelial-to-mesenchymal transition (EMT) (Song, 2007) and SMAD4 is a powerful tumor suppressor in pancreatic tumors (Herman et al., 2013). In the etiopathogenesis of neurological disorders the role of SMAD3/TGFβ signal is not so clear; TGFβ signaling disruption is correlated with several motor neuronal diseases, because of the neuroprotective and anti-inflammatory effects of this pathway (Katsuno et al., 2011). Its overactivation is associated with the formation of β-amyloid plaques in Alzheimer's disease (Town et al., 2008). TGFβ seems to be involved in glial differentiation and production of extracellular matrix (ECM) components for the scaffolding of neurons in the neural tube. Moreover, it is also a neurotropic factor that stimulates neurogenesis and axon growth (Gomes et al., 2005).

Dennler et al. have found specific binding sequences for SMAD3 in the hPAI gene promoter (Dennler et al., 1998). These sequences (so-called CAGA box) are specifically recognized by the SMAD3/SMAD4 complex. Due to an intrinsic steric hindering, SMAD2 cannot interact directly with CAGA box (Dennler et al., 1998). Taking advantage of this specificity, we have developed transgenic reporter lines containing multimerized "CAGA box" to study *in vivo* Smad3-mediated signaling. Genetic, pharmacological and molecular analyses show that in these transgenic lines the reporter gene is activated in a Smad3/TGFβ-responsive manner. During embryo development, reporter expression was mainly found in the central nervous system (CNS). In order to determine the role of TGFβ in neural development, we have performed a series of experiments using the Smad3-responsive line crossed with transgenic fish lines reporting different stages of the progenitor to precursor development. To take advantage of all potentialities of transgenic lines in reporting fluorescent signal dynamics, the majority of analyses have been performed *in vivo*. Results show that postmitotic activation of TGFβ in neural cells controls the progenitor to precursor transition. Finally, we predict that this line might be a valuable tool in drug screening as well as in regeneration and cancer research.

Materials and methods

Animals

Animals were staged and fed as described by Kimmel et al. (1995). The project was examined and approved by the Ethics Committee of the University of Padua with protocol number 18746. *one-eyed pinhead* (*oep*^{m134}) (Schier et al., 1996) and *chordin* (*din*^{tt250}) (Schulte-Merker et al., 1997) mutant carriers were identified both by PCR analysis and phenotype screening of their offspring at 24 hpf. For *oep* PCR screening, the following primers were used: oepm134-wtFw (5′-GGCTCC CTCAGAACACTGTC-3′), oepm134-mutRv (5′-GGCTCCCTCAGAACACT GTA-3′) and oepm134-Rv (5′-CTCTTGGGCACAAAAGAGAA-3′). For *dino* PCR screening, the following oligonucleotides were used: dino-Fw (5′-GACACAAATGCGGGGTAAAC-3′), dino-Rv (5′-ATGTTGCAACTCAG-CAGCAG-3′), dino-wtRv (5′-CTGTGCACAACTCAC-3′) and dino-mutRv (5′-ACTGTGCACAACTCAC-3′). For functional *in vivo* studies we used the following transgenic lines: Tg(*ngn1:GFP*)sb1, Tg(*mnx1:GFP*)ml2. For *neuroD*, we used the Tg(*−2.4 kb neurod:EGFP*) line previously produced in our lab (see also Ronneberger et al., 2012): briefly, the 2.4 kb promoter of zebrafish NeuroD coding gene was cloned in the pG1 vector and the resulting linearized plasmid injected in fertilized eggs. The F1 progeny was screened for GFP expression in the CNS. For *smad7* overexpression, we used the Tg(*hsp70:smad7-YFP*) line (not published, see below). For all the described experiments, heterozygous embryos and larvae were used.

Generation of Tg(12xSBE:EGFP)ia16 and Tg(12xSBE:nls-mCherry)ia15 lines

12 repeats of a Smad3-binding sequence, so called CAGA box (Dennler et al., 1998), were amplified together with major late promoter Adenovirus (MLP) with the attB4cagafor (5′-GGGGA-CAACTTTGTATAGAAAAGTTGGCCCGGGCTCGAGAGCCAG-3′) and attB1-cagarev (5′-GGGGACTGCTTTTTTGTACAAACTTGTTGGAAGAGAGTGAGG ACGAA-3′) oligonucleotides and then cloned into a pDONOR™ P4-P1R