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TAK1 promotes BMP4/Smad1 signaling via inhibition of erk MAPK: A new link in the FGF/BMP regulatory network

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ABSTRACT

FGFs and BMPs act in concert to regulate a wide range of processes in vertebrate development. In most cases, FGFs and BMPs have opposing effects, and specific developmental outcomes arise out of a balance between the two growth factors. We and others have previously demonstrated that signaling pathways activated by FGFs and BMPs interact via inhibitory crosstalk. Here we demonstrate a role for the BMP effector TGF-β Activated Kinase 1 (TAK1) in the maintenance of Smad1 activity in *Xenopus* embryos, via the inhibition of erk MAPK. Up- or downregulation of TAK1 levels produces an inverse alteration in the amount of activated erk MAPK. The inhibition of erk MAPK by TAK1 is mediated by p38 and a corresponding decrease in phosphorylation of MEK. TAK1 morphant embryos show a decrease in the nuclear accumulation of Smad1. Conversely, reduction of erk MAPK activity via overexpression of MAP Kinase Phosphatase1 (MKP1) leads to an increase in nuclear Smad1. Both TAK1 morphant ectoderm and ectoderm treated with FGF show a decrease in the expression of several Smad1-inducible genes. Neural-specific gene expression is inhibited in isolated ectoderm coexpressing noggin and TAK1, suggesting that TAK1 is sufficient to inhibit neural specification. Introduction of TAK1 morpholino oligonucleotide expands the expression of organizer genes, disrupts formation of the boundary between organizer and non-organizer mesoderm, and increases the spatial range of MAPK activation in response to localized FGF. Our results indicate that inhibitory interactions between FGF and BMP4 effector pathways increase the robustness of BMP signaling via a feed-forward mechanism.

1. Introduction

Fibroblast Growth Factors (FGFs) and Bone Morphogenetic Proteins (BMPs) act in concert to regulate many processes in vertebrate development, including patterning of ectodermal placodes (Kwon et al., 2010), cell proliferation and apoptosis in the developing heart (Zhao and Rivkees, 2000), specification of epicardium *vs.* myocardium (van Wijk et al., 2009), formation of mammalian paraxial mesoderm (Miura et al., 2006), partitioning of respiratory and sensory epithelia within the avian olfactory placode (Maier et al., 2010), and vertebrate ectodermal specification (*e.g.*, Marchal et al., 2009; reviewed in Rogers et al., 2009). In nearly all cases, FGFs and BMPs promote opposing developmental outcomes, and the choice of fate for individual cells reflects the prevalence of one type of ligand over another in a specific region during a limited interval.

The FGF and BMP signaling pathways interact antagonistically via inhibitory crosstalk. Since the demonstration that Extracellular Signal-regulated Kinase Mitogen Activated Protein Kinase (erk MAPK) phosphorylates the BMP effector Smad1 in the linker region, thus inhibiting nuclear translocation (Kretzschmar et al., 1997), this interaction has been shown to play a role in axial patterning (Sater et al., 2003) and neural specification (Pera et al., 2003) in *Xenopus*, and in primordial germ cell formation in mouse embryos (Aubin et al., 2004). During vertebrate ectodermal specification, high levels of BMP signaling lead to epidermal development, while inhibition of BMP signaling or activation of erk MAPK by FGFs or Insulin-Like Growth Factor (IGF) (Richard-Parpaillon et al., 2002, Pera et al., 2003) promotes neural specification. Morpholino oligonucleotide-mediated knockdown of three major BMP antagonists (noggin, chordin, and follistatin) inhibits formation of the neural plate (Khokha et al., 2005), demonstrating that inhibition of BMP signaling is required for neural specification *in vivo*.

Our earlier studies demonstrated that BMP/TGF-β Activated Kinase 1 (TAK1) signaling reduces erk MAPK activity during

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Xenopus ectodermal specification (Goswami et al., 2001). The inhibition of activated erk MAPK by TAK1 suggests that TAK1 may indirectly promote Smad1 activity by downregulating erk MAPK activity. We undertook the following studies to determine whether TAK1 contributes to Smad1 function and the overall robustness of the BMP pathway via its inhibitory effects on erk MAPK.

2. Methods

2.1. Microinjection and microsurgery

Preparation of embryos, microsurgical manipulations, and treatment of isolated ectoderm with purified recombinant *Xenopus* bFGF were performed as described (Uzgare et al., 1998). For overexpression studies, synthetic mRNA was prepared by *in vitro* transcription of linearized plasmid DNA, using the mMessage Machine *in vitro* transcription system (Ambion/Applied Biosystems). Unless otherwise indicated, approximately 10 nl of mRNA or morpholino oligonucleotide (MO) (Gene Tools, Inc., Summerton, OR) stock at the appropriate concentration was injected near the animal pole into each blastomere at the 2-cell stage.

A 1 mM stock of SB203580 (Sigma; St. Louis, MO) was prepared in dimethyl sulfoxide (DMSO) and stored at −20°. This stock was added to 1/3 MMR for a final concentration of 10 μM immediately before use.

For FGF treatments, tissue isolates were held in Very Low Ca^{+2} Mg^{+2} Modified Ringer (VLCMR; Lamb and Harland, 1995) to inhibit healing; this medium contained either 800 ng/ml Bovine Serum Albumin (BSA) or 400 ng/ml BSA+400 ng/ml recombinant human basic fibroblast growth factor (bFGF) (Roche Applied Sciences, Indianapolis, IN). For FGF bead treatments, beads were prepared as described in Cohn et al. (1995): briefly, Affi-Gel Heparin beads (Bio-Rad, Hercules, CA) were incubated in 10 μg/ml bFGF or PBS for 1 h and then transferred to the inner surface of an ectodermal isolate. Beads were held in place with a coverslip fragment supported by modeling clay.

2.2. Cell fractionation

A minimum of 20 embryos was lysed in 100 μl Kinase Buffer (20 mM HEPES, 40 mM $MgCl_2$, 20 mM EGTA, 1 mM DTT, 80 mM glycerol 2-phosphate, 50 mM NaF, 1 mM Na Orthovanadate, 1 mM microcystin, Roche Protease Inhibitor Cocktail [Roche Applied Sciences]; Uzgare et al., 1998). The lysate was homogenized by repeated pipetting and incubated on ice for 10 min. It was then centrifuged 500 g for 10 min at 4°, and the supernatant was then recentrifuged at 5500 g for 20 min at 4° to separate the nuclear and cytoplasmic fractions.

2.3. Immunoblots

Lysates or cellular fractions were separated by SDS/PAGE under standard conditions and then transferred to nitrocellulose via a semi-dry transfer system. The resulting blots were rinsed in TBST, blocked, incubated with primary and HRP-conjugated secondary antibodies, and processed for chemiluminescent detection (ECL system, Amersham/GE Healthcare Bio-Sciences, Piscataway, NJ). Antibodies were used at the following concentrations: rabbit anti-Smad1 (AbCam, Cambridge MA) 1:1000; rabbit anti-p38 (Cell Signaling Technology [CST], Beverly MA) 1:1000; rabbit anti-phospho-p38 (CST) 1:500; mouse anti-diphospho-erk MAPK (Sigma) 1:2000; erk MAPK (CST) 1:1000; rabbit anti-MEK 1/2 (CST) 1:1000; rabbit anti-phospho-MEK1/2 (CST) 1:500; rabbit anti-TAK1 (Abcam) 1:1000; rabbit anti-tubulin (Abcam) 1–1000; mouse anti-nuclear Lamin B2 (Abcam) 1:100. Quantification of immunoblots was carried out using Image J (Abramoff et al., 2004), a Java-based image processing program similar to NIH Image for the Macintosh platform. Immunoblots shown in Figs. 1–6 are unprocessed images; all immunoblots used for quantification were visualized with exposures in the linear range, which may appear relatively light.

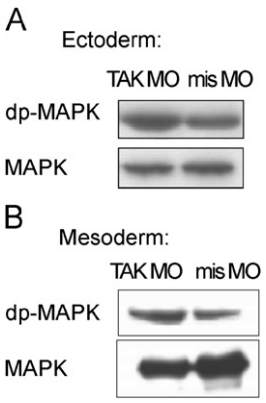

Fig. 1. Increase in ERK MAPK phosphorylation following TAK1 knockdown. Embryos were injected with 20 ng of either the TAK1 MO (TAK MO) or the 5-base mispair (mis MO). Tissues were isolated when embryos reached early gastrula (10–10.25). (A) Activated (diphospho; dp-MAPK) and total ERK MAPK (MAPK) from isolated ectoderm. (B) Activated (diphospho; dp-MAPK), and total ERK MAPK (MAPK) from isolated mesoderm. This mesoderm consists of the lower (*i.e.*, vegetalward) marginal zone minus the superficial layer, around the entire circumference of the embryo. (20 tissue isolates/sample; $N=3$ independent experiments).

2.4. RT-PCR

For radiolabeled RT-PCR, RNA isolation, preparation of cDNA, and polymerase chain reaction (PCR) assays were carried out as described (Uzman et al., 1998). The RT-PCR products were separated on 6% polyacrylamide gels and visualized by autoradiography.

2.5. Q-RT-PCR

Embryos were lysed in Trizol (Invitrogen, Carlsbad, CA); RNA was isolated according to manufacturer's instructions and reverse transcribed using either the Superscript III First-Strand Synthesis kit (Invitrogen) or the First-Strand cDNA Synthesis kit (Roche Applied Sciences). The resulting cDNA was assayed by PCR using BioRad iQ SYBR Green Supermix (BioRad) with an ABI Prism 7000 thermal cycler, according to manufacturer's instructions. Differences in transcript levels were evaluated by the Comparative C_t method (Livak and Schmittgen, 2001) using ornithine decarboxylase (ODC) for normalization. "Fold-changes" were calculated as the ratio of experimental value/control value.

PCR primers included BMP-4 (sequences from Fainsod et al., 1994), GATA4 (Afouda et al., 2005), Xvent1 (Sander et al., 2007), and NCAM (Yokota et al., 2003). All primers were obtained from Bioneer (Alameda, CA).

2.6. In situ hybridization and immunohistochemistry

Digoxigenin-labeled RNA probes for were prepared using the Maxiscript *in vitro* transcription kit (Ambion/Applied Biosystems). *In situ* hybridization was carried out as described in Patil et al., (2006). Fixation and immunohistochemistry for diphospho-ERK MAPK was carried out via a modification of methods described in Gabay et al. (1997): after exposure to FGF beads, ectodermal