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Syndecan4 coordinates Wnt/JNK and BMP signaling to regulate foregut progenitor development

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ABSTRACT

Temporally and spatially dynamic Wnt and BMP signals are essential to pattern foregut endoderm progenitors that give rise to the liver, pancreas and lungs, but how these two signaling pathways are coordinated in the extracellular space is unknown. Here we identify the transmembrane heparan sulphate proteoglycan Syndecan-4 (Sdc4), as a key regulator of both non-canonical Wnt and BMP signaling in the *Xenopus* foregut. Foregut-specific Sdc4 depletion results in a disrupted Fibronectin (Fn1) matrix, reduced cell adhesion, and failure to maintain foregut gene expression ultimately leading to foregut organ hypoplasia. Sdc4 is required to maintain robust Wnt/JNK and BMP/Smad1 signaling in the *hhex*+ foregut progenitors. Pathway analysis suggests that Sdc4 functionally interacts with Fzd7 to promote Wnt/JNK signaling, which maintains foregut identity and cell adhesion. In addition, the Sdc4 ectodomain is required to support Fn1 matrix assembly, which is essential for the robust BMP signaling that promotes foregut gene expression. This work sheds lights on how the extracellular matrix can coordinate different signaling pathways during organogenesis.

1. Introduction

The endoderm germ layer gives rise to the epithelial lining of the digestive and respiratory tracts and associated organs such as liver, pancreas, and lungs. Shortly after gastrulation a series of growth factor signals from adjacent mesoderm progressively patterns the naïve embryonic endoderm along the anterior-posterior (A-P) axis and to induce various organ lineages in the primitive gut tube (Horb and Slack, 2001; Zaret, 2008; Zorn and Wells, 2009). These paracrine signals are highly dynamic, with the same factors having dramatically different impacts on organogenesis at different times or at different positions along the embryonic gut tube (McLin et al., 2007; Wandzioch and Zaret, 2009). The mechanisms that coordinate these signaling dynamics *in vivo* are poorly understood.

Previous studies in *Xenopus* have shown that precise levels of Wnt and BMP during late gastrula and early somite stages are critical to pattern and maintain foregut versus hindgut progenitors. At this stage in development a combination of low-Wnt and high-BMP are required to maintain the ventral foregut progenitors, which express the homeobox gene *hhex*, whereas high-Wnt and high-BMP promotes hindgut progenitors (Kenny et al., 2012; McLin et al., 2007). Differential Wnt activity is controlled in part by the secreted Wnt-antagonist Sfrp5, which protects anterior endoderm from posterior Wnt ligands (Li et al., 2008). As a result the foregut cells experience a low, but critical level of both Wnt/β-catenin and Wnt/JNK signaling through Wnt receptor the Frizzled 7 (Fzd7) to coordinate foregut identity and morphogenesis (Zhang et al., 2013). If Wnt activity is too high, such as with the loss of Sfrp5 or Wnt overexpression, the anterior endoderm adopts a hindgut rather than foregut fate (Li et al., 2008; McLin et al., 2007). On the other hand, if Wnt activity in the foregut is too low, such as in Fzd7 depleted embryos, the foregut progenitors arrest with disrupted cell morphology and fail to maintain *hhex* expression resulting in foregut organ agenesis (Zhang et al., 2013).

During the same period of development, BMP ligands secreted from the cardiac mesoderm are required to maintain ventral foregut progenitors in *Xenopus*. Recent studies indicate that this BMP signaling is dependent upon the extracellular Fibronectin (Fn1) matrix that forms between the endoderm and the adjacent mesoderm (Kenny et al., 2012). Current evidence suggests that a functional balance between secreted Tolloid-like (Tll) metalloproteinases and the Tll-inhibitor Sizzled (Szl) modulate the deposition of Fn1 fibrils and that the Fn1-rich extracellular matrix

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(ECM) promotes robust BMP signaling, perhaps by increasing the local ligand concentration in the foregut microenvironment, which is critical for liver, pancreas, and lung organogenesis (Kenny et al., 2012).

Exactly how the ECM coordinates Wnt and BMP activity in the foregut is still poorly understood. Although genetic and biochemical studies (primarily in *Drosophila* and cell culture) indicate that the ECM is an important regulator of growth factor bioavailability and may provide a co-receptor function; in most cases the *in vivo* molecular mechanisms are still obscure (Hacker et al., 2005; Lin, 2004). In particular the role of Fn1, a vertebrate specific ECM protein, is still poorly defined. The transmembrane heparan sulphate proteoglycan (HSPG) Syndecan 4 (Sdc4) has emerged as a candidate for coordinating extracellular Wnt and BMP signaling in the *Xenopus* foregut. Sdc4 together with Integrin-α5β1 are well known to promote Fn1 matrix assembly and are key focal adhesions components that recruit other ECM proteins (Dzamba et al., 2009; Morgan et al., 2007; Ramirez and Rifkin, 2009; Schwarzbauer and DeSimone, 2011). In some tissues Syndecans act as co-receptors for various growth factors and can transduce signals *via* their intracellular domains (Alexopoulou et al., 2007). Notably Sdc4-Fn1 complexes can function as Fzd7 co-receptors to promote non-canonical Wnt signaling *via* Jun N-terminal Kinase (JNK) and Rho family of small GTPases in certain cellular contexts including: *Xenopus* gastrulation, neural crest migration and murine muscle cell homeostasis (Bentzinger et al., 2013; Davidson et al., 2006; Escobedo et al., 2013; Matthews et al., 2008; Munoz et al., 2006; Ohkawara et al., 2011).

In this study we tested the hypothesis that Sdc4 might coordinate Fn1-regulated Wnt and BMP signaling in the *Xenopus* embryonic foregut. Using foregut specific depletion we show that Sdc4 is required to maintain foregut progenitor identity, proliferation and morphogenesis. Our data suggest that Sdc4 works together with Fzd7 to transduce Wnt/JNK signals that maintain foregut gene expression and cell-cell adhesion. In addition, Sdc4 is required for Fn1 matrix deposition at the boundary between foregut endoderm and BMP-expressing cardiac mesoderm. Loss of the Fn1 matrix in Sdc4-depleted embryos disrupts a positive BMP-feedback loop that is critical for maintaining the foregut progenitors. Together these data provide a paradigm for how Sdc4 regulation of the ECM might coordinate Wnt and BMP signaling in many development and disease contexts.

2. Material and methods

2.1. Embryo manipulations and microinjections

Xenopus laevis embryo manipulations were performed as previously described (McLin et al., 2007) and staged based on the Nieuwkoop and Faber normal table of development (Nieuwkoop, 1994). To specifically target the foregut endoderm and avoid the chordomesoderm, antisense morpholino oligos (MO) and/or mRNAs (along with a fluorescent lineage tracer) were microinjected into D1 cells of 32-cell stage embryos (Moody, 1987). To knockdown Sdc4, previously validated antisense MOs were used at either a total amount of 25 or 50 ng (Matthews et al., 2008; Munoz et al., 2006; Ohkawara et al., 2011). Other MOs were: Fzd7-MOs (25 or 50 ng) (Sumanas and Ekker, 2001); Szl-MO (20 ng) (Collavin and Kirschner, 2003; Lee et al., 2006), Fn1 MOs:Fn1.S-MO+Fn1.L-MO (25 ng each) (Davidson et al., 2006) and Cdh3 MO (Ninomiya et al., 2012). For all experiments the total amount of MO injected is equalized by addition of the standard control MO (GeneTools) as necessary. Synthetic mRNA was generated from the following plasmids: pCS2+Sdc4 (Munoz et al., 2006), pCS2+Sdc4ΔPBM (kindly provided by Dr. J. Larrain) (Carvallo et al., 2010), and pCS2+c.a.JNK (Liao et al., 2006). Recombinant hBMP2 (10 μM, 15 nl; R&D Systems) or Wnt11 protein (10 μM, 20 nl; R&D Systems) was injected into the closing blastocoel at stage 13. The following cell-soluble inhibitors were dissolved in DMSO and added to the media at stage 12: BMP-inhibitor LDN 193189 (100 μM, Axon Medchem), FGF-inhibitor PD173074 (300 μM; TOCRIS), and FGF-inhibitor SU5402 (10 μM with 0.1 M ATP). All injection and inhibitor experiments were repeated at least three independent times with similar results and a representative example is shown.

2.2. In situ hybridization and immunohistochemistry

In situ hybridization and confocal immunohistochemistry were performed as previously described (Zhang et al., 2013). Details on the antisense RNA probes used are available upon request. The following primary antibodies were used: rabbit anti-β-catenin (1:250; H-102, Santa Cruz Biotechnologies), mouse anti-Cdh3 (1:200; 6B6, DSHB), mouse anti-β1-Integrin (1:500; 8C8, DSHB), rabbit anti-phospho-HistoneH3 (1:250; Cell signaling), rabbit anti-active-JNK (1:250; Promega), anti-Fibronectin (1:250; 4H2; gift from Dr. DeSimone) and rabbit anti-phospho-Smad 1/5/8 (1:250; Cell signaling). The following secondary antibodies were used: goat anti-rabbit-cy5, goat anti-rabbit-cy2 or goat anti-mouse-cy5 (1:300; Jackson Immunoresearch). Nuclei were counterstained with Topro-3. In all experiments, exactly the same confocal and camera settings were used for control and manipulated sibling embryos.

Image-J was used to quantify the pixel intensity of nuclear pSmad1/5/8, pJNK and nuclear β-catenin immunostaining in the MO-targeted foregut endoderm cells (50–150 cells per foregut), using 15 μm mid-sagittal optical sections of five stage 19 embryos for each condition. A fluorescent lineage tracer was used to identify the injected cells and Topro-3 staining was used to define the nucleus from surrounding cytoplasm and to normalize the antibody intensity. The average normalized pixel intensity (in arbitrary units) was calculate for each condition +/− standard deviation. Repeated measure ANOVA with covariance structure using R software was used to assess statistically significant differences in mean pSmad1/5/8, pJNK and nuclear β-catenin intensity between the foregut endoderm cells in different condition compared to controls. To calculate the average mitotic index at stage 11, we count the total number of phospho-Histone H3 positive nuclei in the anterior endoderm, divided by total number of anterior endoderm cells (~100 cells) or-# for each experimental groups (n=4 embryos/condition). All experiments were repeated three times with similar results.

2.3. BRE:Luc, TOP:flash and ATF2:luciferase assay

BMP/pSmad1, Wnt/β-catenin and Wnt/JNK activity in the foregut were assayed using transcriptional reporter assays. BRE: Luciferase (150 pg) (von Bubnoff et al., 2005), Top-flash (150 pg) (Korinek et al., 1997) or ATF2:luciferase, plasmids (van Dam et al., 1995) co-injected with pRL-TK:renilla (25 pg) into D1 presumptive foregut cells of 32-cell stage embryos. At stage 19 extracts were prepared from each condition in triplicate using five embryos per replicate, and luciferase activity was measured using a commercial kit (Promega). Luciferase activity was normalized to co-injected TK:renilla and the mean relative activity of the triplicate samples was shown ±S.D. with pairwise student *t*-tests to determine significant differences in expression. Each experiment was repeated a minimum of three times and a representative result is shown.

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