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Spatial regulation of cell cohesion by Wnt5a during second heart field progenitor deployment

Ding Li [a], Tanvi Sinha [a], Rieko Ajima [b], Hwa-Seon Seo [a], Terry P. Yamaguchi [c], Jianbo Wang [a,*]

[a] Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL 35294, USA
[b] Mammalian Development Laboratory, National Institute of Genetics, Yata 1111, Mishima 411-8540, Japan
[c] Cancer and Developmental Biology Laboratory, NCI-Frederick, NIH, Frederick, MD 21702, USA

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ABSTRACT

Wnt5a, a non-canonical Wnt ligand critical for outflow tract (OFT) morphogenesis, is expressed specifically in second heart field (SHF) progenitors in the caudal splanchnic mesoderm (SpM) near the inflow tract (IFT). Using a conditional *Wnt5a* gain of function (GOF) allele and *Islet1-Cre,* we broadly over-expressed Wnt5a throughout the SHF lineage, including the entire SpM between the IFT and OFT. Wnt5a over-expression in *Wnt5a* null mutants can rescue the cell polarity and actin polymerization defects as well as severe SpM shortening, but fails to rescue OFT shortening. Moreover, Wnt5a over-expression in wild-type background is able to cause OFT shortening. We find that *Wnt5a* over-expression does not perturb SHF cell proliferation, apoptosis or differentiation, but affects the deployment of SHF cells by causing them to accumulate into a large bulge at the rostral SpM and fail to enter the OFT. Our immunostaining analyses suggest an inverse correlation between cell cohesion and Wnt5a level in the wild-type SpM. Ectopic Wnt5a expression in the rostral SpM of *Wn5a-GOF* mutants diminishes the upregulation of adherens junction; whereas loss of Wnt5a in *Wnt5a* null mutants causes premature increase in adherens junction level in the caudal SpM. Over-expression of mouse Wnt5a in *Xenopus* animal cap cells also reduces C-cadherin distribution on the plasma membrane without affecting its overall protein level, suggesting that Wnt5a may play an evolutionarily conserved role in controlling the cell surface level of cadherin to modulate cell cohesion during tissue morphogenesis. Collectively, our data indicate that restricted expression of Wnt5a in the caudal SpM is essential for normal OFT morphogenesis, and uncover a novel function of spatially regulated cell cohesion by Wnt5a in driving the deployment of SHF cells from the SpM into the OFT.

1. Introduction

The heart arises from progenitor cells located in the crescent-shaped (in the mouse) or bilateral (in the chick) cardiogenic fields in the anterior lateral plate mesoderm (Dyer and Kirby, 2009; Evans et al., 2010). Embryo folding brings the lateral portions of the cardiogenic field to the ventral midline, allowing them to fuse and form the initial heart tube. Commonly referred to as the first heart field (FHF), the progenitor cells contributing to the initial heart tube differentiate early to give rise primarily to the left ventricle (LV) and atria. On the other hand, the progenitor cells residing in the more medial portion of the cardiogenic fields are shifted dorsally and remain undifferentiated during the initial heart tube formation, and are referred to as the second heart field (SHF). The SHF extends from the rostral pharyngeal mesoderm (PM) to the caudal splanchnic mesoderm (SpM), and is attached to heart tube only at its arterial (outflow) and venous (inflow) poles. SHF progenitors undergo extensive proliferation, and gradually differentiate and deploy into the heart tube to form the right ventricle (RV) and the outflow tract (OFT) at the arterial pole, and part of the atria and atrial septum at the venous pole (Dyer and Kirby, 2009; Evans et al., 2010; Vincent and Buckingham, 2010).

The myocardial cells within the early heart tube undergo prolonged proliferation arrest (van den Berg et al., 2009). The elongation of the heart tube, therefore, is driven primarily by addition of cells from the SHF. Sufficient elongation is necessary for cardiac morphogenesis such as rightward looping of the heart, and aligning the OFT over the inter-ventricular septum so that upon cardiac neural crest invasion, the OFT can be properly septated into the aorta and pulmonary artery and connected to the LV and RV, respectively. Aberrant OFT morphogenesis can cause a spectrum of conotruncal defects such as double outlet right ventricle (DORV), overriding aorta, transposition of the great arteries, pulmonary atresia and persistent truncus arteriosus (PTA) (Dyer and Kirby,

* Corresponding author.
E-mail address: j18wang@uab.edu (J. Wang).

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2009; Evans et al., 2010; Vincent and Buckingham, 2010).

Extensive studies in the field have delineated the signaling pathways and transcriptional networks that orchestrate cell proliferation and differentiation in the SHF to generate sufficient number of cardiomyocytes for the heart (Black, 2007; Vincent and Buckingham, 2010; Xin et al., 2013). However, relatively little is known about how SHF cells are deployed into the heart. Our previous work has implicated a role of the non-canonical Wnt/planar cell polarity (PCP) pathway in the deployment of SHF cells to the OFT. Initially identified in *Drosophila*, the PCP pathway coordinates cellular polarity in the plane of the epithelium, and regulates polarized cell behavior such as oriented cell intercalation and directional cell migration during convergent extension (CE) morphogenesis to modulate tissue shape and dimensions in vertebrates (Devenport, 2014; Goodrich and Strutt, 2011; Tada and Heisenberg, 2012; Zallen, 2007). The PCP pathway does not lead to β-catenin stabilization, but it utilizes some components of the canonical Wnt pathway, including the Frizzled (Fz) receptor and cytoplasmic protein Dishevelled (Dvl/Dsh), to function together with a set of distinct core PCP proteins like Van Gogh (Vang/Vangl). PCP signaling has been reported to regulate a diverse array of effectors, such as JNK, GTPase Rho/Rac/Cdc42/Rab5, and formin protein Daam1, and function in a context- and tissue-specific manner to modulate cytoskeletal dynamics, cell cohesion, protrusive activity, *etc.* (Devenport, 2014; Gray et al., 2011; Habas et al., 2001; Wang et al., 2012). Studies in *Xenopus* and zebrafish have identified two non-canonical Wnts, Wnt5a and Wnt11, as the ligands that activate PCP signaling during tissue morphogenesis.

In the mouse, we and others have carried out tissue-specific gene ablation to demonstrate that PCP genes *Dvl1/2* and *Vangl2* are required specifically in the SHF lineage for OFT elongation and morphogenesis (Ramsbottom et al., 2014; Sinha et al., 2012). Furthermore, loss of either *Wnt5a* or *Wnt11* also leads to severe conotruncal defects in mice (Schleiffarth et al., 2007; Zhou et al., 2007). Lineage tracing studies have revealed that *Wnt11* starts to be expressed in the rostral SHF cells in the PM, and *Wnt11*-lineage populates the superior myocardial wall of the OFT and contributes specifically to the sub-aortic myocardium (Sinha et al., 2015b). Conversely, *Wnt5a* is expressed in the caudal SHF cells in the SpM, and loss of *Wnt5a* specifically diminishes the inferior myocardial wall of the OFT and its derivative, the sub-pulmonary myocardium (Sinha et al., 2015a). SHF progenitors in the caudal SpM of *Dvl1/2* and *Wnt5a* null mutants display no cell proliferation or apoptosis defects, but lack the normal elongated and protrusive morphology, display defective actin organization, and are aggregated into compact of clusters instead of organizing into a cohesive sheet like the wild-type cells. These observations lead us to propose the first model on SHF deployment, in which Wnt5a, acting through PCP, induces oriented cell intercalation to incorporate SHF progenitors into a cohesive sheet at the caudal end of the SpM, thereby creating a pushing force to deploy SHF cells rostrally into the OFT (Sinha et al., 2015a, 2012).

In the current study, we attempted to explore additional mechanisms underlying SHF deployment by taking a gain-of-function approach to over-express Wnt5a broadly in the SHF lineage. Our results not only provide additional support to our original model, but more importantly uncover an additional role of restricted Wnt5a expression to spatially regulate cell cohesion to drive the deployment of SHF cells from the SpM into the OFT.

2. Methods

2.1. Mouse strains

Rosa26^{Wnt5a} mice were generated as described (Cha et al., 2014) and illustrated in Fig. 1A. A three-primer PCR strategy (Rosa10: CTCTGCTGCCTCCTGGCTTCT; Rosa11: CGAGGCGGATCACAAGCAATA and R26R2; GCGAAGAGTTTGTCCTCAACC) was used to genotype *Rosa26*^{Wnt5a} mice. Genotyping of *Wnt5* and *Islet1-Cre* were described previously (Cai et al., 2003; Yamaguchi et al., 1999).

Animal care and use was in accordance with NIH guidelines and was approved by the Animal Care and Use Committee of the University of Alabama at Birmingham.

2.2. Embryo collection, staining and imaging

Embryos were dissected at appropriate embryonic stages and yolk sacs were retained for genotyping. Embryos were fixed in 4% paraformaldehyde at 4 °C overnight and stored in PBS until further use.

For OFT length measurement and quantification, E9.5 embryos with comparable number of somites were imaged and analyzed as previously described (Sinha et al., 2012).

For immunostaining, fixed embryos were cryo-embedded and cut to 10 μm sections. Sections were blocked with 5% normal donkey serum in PBS followed by primary antibodies incubation overnight. The primary antibodies used in this study are: rabbit anti-GFP (A-11122, Invitrogen), rat anti-Wnt5a (MAB645, R&D Systems), mouse MF20 (DSHB), rat anti-N-cadherin (MNCD2, DSHB), mouse anti-α-catenin (610193, BD Transduction laboratories) and Rabbit anti-pHH3 (06-570, Millipore). Afterwards, sections were incubated with appropriate fluorescent secondary antibodies or FITC conjugated Phalloidin (Sigma) prior to be mounted with Vectashield DAPI medium (Vector Laboratory). Images were acquired with an Olympus FV1000 Laser Confocal Scanning microscope and analyzed with the FV10-ASW software. LWR analysis and angular measurement of SHF cells in the caudal SpM were performed as previously described (Sinha et al., 2015a).

In situ hybridization was performed following a previously described protocol (Sinha et al., 2015b).

2.3. Wholemount immunostaining and 3D reconstruction

SpM along with part of the distal OFT was dissected out from fixed embryos (Fig. 4A). Samples were blocked with 5% normal donkey serum in PBS and incubated with primary antibody (MF20) at 4 °C overnight, washed and stained with fluorescent secondary antibody. Afterwards, samples were placed in an agarose plate with the ventral side facing up, and imaged by confocal microscope. Z-stack images were imported into ImageJ for 3D reconstruction.

2.4. EdU pulse-chase labeling, detection and wholemount imaging

Pregnant female mice were injected with 250 μg of EdU (5-ethynyl-2′-deoxyuridine, Invitrogen) intraperitoneally at E9.25 to pulse label the proliferating cells. To detect the region where cells were proliferating, injected mice were sacrificed two hours later to harvest embryos for wholemount EdU detection according to the user manual from the manufacturer (Invitrogen). To chase the EdU pulse-labeled proliferating cells, female mice were injected with 2.5 mg thymidine (Sigma) after the two-hour EdU pulsing period, and were sacrificed 15 h later to harvest embryos at E10.0. After EdU detection, embryos were processed for MF20 wholemount immuno-staining before the SpM and OFT were dissected out for imaging by confocal microscopy. Z-stack images were imported into ImageJ for 3D reconstruction.

2.5. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

SpM micro-dissected from three E9.5 control or mutant embryos was used for RNA extraction with Trizol. cDNA was synthesized and used for semi-quantitative PCR as described previously (Wang et al., 2011).