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Mapping the dynamic expression of *Wnt11* and the lineage contribution of *Wnt11*-expressing cells during early mouse development

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ABSTRACT

Planar cell polarity (PCP) signaling is an evolutionarily conserved mechanism that coordinates polarized cell behavior to regulate tissue morphogenesis during vertebrate gastrulation, neurulation and organogenesis. In *Xenopus* and zebrafish, PCP signaling is activated by non-canonical Wnts such as Wnt11, and detailed understanding of *Wnt11* expression has provided important clues on when, where and how PCP may be activated to regulate tissue morphogenesis. To explore the role of *Wnt11* in mammalian development, we established a *Wnt11* expression and lineage map with high spatial and temporal resolution by creating and analyzing a tamoxifen-inducible *Wnt11-CreER* BAC (bacterial artificial chromosome) transgenic mouse line. Our short- and long-term lineage tracing experiments indicated that *Wnt11-CreER* could faithfully recapitulate endogenous *Wnt11* expression, and revealed for the first time that cells transiently expressing Wnt11 at early gastrulation were fated to become specifically the progenitors of the entire endoderm. During mid-gastrulation, *Wnt11-CreER* expressing cells also contribute extensively to the endothelium in both embryonic and extraembryonic compartments, and the endocardium in all chambers of the developing heart. In contrast, *Wnt11-CreER* expression in the myocardium starts from late-gastrulation, and occurs in three transient, sequential waves: first in the precursors of the left ventricular (LV) myocardium from E7.0 to 8.0; subsequently in the right ventricular (RV) myocardium from E8.0 to 9.0; and finally in the superior wall of the outflow tract (OFT) myocardium from E8.5 to 10.5. These results provide formal genetic proof that the majority of the endocardium and myocardium diverge by mid-gastrulation in the mouse, and suggest a tight spatial and temporal control of *Wnt11* expression in the myocardial lineage to coordinate with myocardial differentiation in the first and second heart field progenitors to form the LV, RV and OFT. The insights gained from this study will also guide future investigations to decipher the role of non-canonical Wnt/PCP signaling in endoderm development, vasculogenesis and heart formation.

Introduction

In mice and humans there are 19 independent *Wnt* genes, encoding secreted glycoproteins that have diverse and critical roles during embryonic development, in adult tissue homeostasis and in human diseases (Willert and Nusse, 2012). Wnt ligands can be broadly divided into two classes: canonical Wnts such as Wnt1 and Wnt3a, and non-canonical Wnts such as Wnt5a and Wnt11. Canonical Wnts bind to Frizzled (Fz) receptors and the Lrp5/6 family of co-receptors to activate cytoplasmic protein Disheveled (Dsh/Dvl), which in turn stabilizes β-catenin to activate gene transcription. Non-canonical Wnts, on the other hand, share certain components with the canonical Wnt pathway such as Fz and Dsh/Dvl, but signal through multiple β-catenin independent branches that include the Wnt/Ca^{2+} and the planar cell polarity (PCP) pathways (Angers and Moon, 2009; MacDonald et al., 2009; van Amerongen and Nusse, 2009; Wallingford et al., 2000).

Of these non-canonical Wnt signaling branches, the PCP pathway has been studied most extensively in various vertebrate and invertebrate model organisms. Initially identified as one of the signaling mechanisms that coordinate cellular polarity in the plane of the epithelium, PCP signaling also regulates polarized cell behavior such as mediolateral (M-L) cell intercalation and

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directional migration during convergent extension (CE) tissue morphogenesis (Keller, 2002; Zallen, 2007). In addition to Fz and Dsh/Dvl, the PCP pathway requires a set of distinct "core" proteins such as the tetraspan membrane protein Van Gogh (Vang/Vangl) and the atypical cadherin Flamingo (Fmi). What functions downstream of these core proteins as PCP effectors remains elusive and is likely to be context- and tissue-dependent, and may include JNK, small GTPase Rho/Rac/Cdc42 and the formin protein Daam1 (Goodrich and Strutt, 2011; Habas et al., 2001; Tree et al., 2002; Wallingford, 2012).

In *Xenopus* and zebrafish, Wnt5a and Wnt11 have been identified as two primary ligands capable of activating non-canonical Wnt/PCP signaling to regulate tissue morphogenesis during gastrulation (Heisenberg et al., 2000; Kilian et al., 2003; Tada and Smith, 2000; Walentek et al., 2013; Wallingford et al., 2001), although in other contexts, they can also activate canonical Wnt signaling (Cha et al., 2008, 2009; Tao et al., 2005). During gastrulation, Wnt11 acts in both cell-autonomous and cell-non-autonomous fashion to regulate polarized cell intercalation and directional migration of mesodermal and endodermal cells (Heisenberg et al., 2000; Ulrich et al., 2003; Witzel et al., 2006). Consequently, perturbing *Wnt11* expression results in failure of axial elongation and midline convergence of foregut endoderm in frog and zebrafish embryos (Heisenberg et al., 2000; Li et al., 2008; Matsui et al., 2005; Tada and Smith, 2000; Walentek et al., 2013).

In the mouse, PCP signaling has so far been implicated in a number of processes such as neurulation and cardiovascular and limb development (van Amerongen, 2012; Wang et al., 2012). PCP-mediated tissue morphogenesis is likely to have an even broader impact on mammalian development and human diseases. Given the essential roles of Wnt5a/Wnt11 in initiating PCP signaling and the fact that they act in paracrine or autocrine fashion, determining their spatial and temporal expression pattern will shed light on where and when PCP signaling is potentially activated, and provide hints on what additional processes and tissues may require PCP function. Indeed, *in situ* hybridization studies of *Wnt5a* expression have led to novel models as to how PCP signaling could be operative in mammals to regulate heart and limb development (Gao et al., 2011; Gros et al., 2010; Sinha et al., 2012; Yamaguchi et al., 1999a).

In comparison, our understanding of *Wnt11* in the mouse is more limited. *In situ* studies indicate that *Wnt11* is expressed first in a scattered pattern around the primitive streak at early gastrulation, and later on in the developing heart, posterior trunk and urogenital system (Kispert et al., 1996). *Wnt11*$^{-/-}$ mouse embryos display no major gastrulation defects, but die in-utero or shortly after birth with multiple cardiac defects (Majumdar et al., 2003; Nagy et al., 2010; Zhou et al., 2007). Due to limited spatial and temporal resolution, the existing RNA *in situ* data do not provide sufficient information as to which cardiac lineage (s) express *Wnt11* and the duration of *Wnt11* expression in that lineage, and therefore the spatio-temporal requirement for Wnt11 in heart development remains to be elucidated. Moreover, *in situ* based expression studies often cannot inform us of the potential fate and lineage of the cells expressing the gene of interest, for instance the scattered *Wnt11*-expressing cells during early gastrulation in the mouse (Kispert et al., 1996).

To overcome the limitations of *in situ* hybridization, and to establish a *Wnt11* expression map with high spatial and temporal resolution and lineage information, we generated a tamoxifen inducible *Wnt11-CreER* transgene using BAC (Bacterial Artificial Chromosome) recombineering technology. Because of their large size (150–300 kb), BAC transgenes have been shown to recapitulate endogenous gene expression patterns (Lee et al., 2001). *CreER-T2* encodes a fusion protein between Cre and a mutated estrogen receptor (ER) (Feil et al., 1997; Leone et al., 2003). CreER is normally sequestered in the cytoplasm by the ER domain. Exposure to tamoxifen leads to a temporary relief in this sequestration, allowing CreER to enter the nucleus and induce recombination. By crossing *Wnt11-CreER* with *Rosa26* Cre reporters (R26R) (Madisen et al., 2010; Soriano, 1999) and administering tamoxifen during gestation, we can transiently induce Cre activation to permanently label *Wnt11* expressing cells and their descendents. By analyses of embryos shortly after tamoxifen-induction, we can establish a high-resolution expression map of *Wnt11-CreER* and compare it with the existing *in situ* data. Alternatively, we can trace the fate of *Wnt11-CreER* expressing cells by collecting and analyzing embryos after a more extended period.

In the current study, we focused on using our *Wnt11-CreER* BAC transgene to perform detailed expression and lineage analyses during gastrulation and heart development. Our results uncovered strikingly specific and dynamic expression of *Wnt11* in progenitors of the endodermal and endothelial lineages during early and mid-gastrulation. In the heart, we demonstrated that not only did the *Wnt11-CreER* expressing cells contribute to the three major cardiac lineages (the endocardium, myocardium and epicardium), but they did so in a highly spatio-temporally controlled fashion. Collectively, our results provide novel and significant insights and open up multiple avenues to explore the involvement of Wnt11/PCP signaling during early endoderm development, vasculogenesis and heart formation.

Materials and methods

Cloning and BAC recombineering to generate Wnt11-CreER BAC transgenic mice

To create an efficient system to target the tamoxifen-inducible CreER T2 into BACs, we cloned the CreER T2 fragment into the pIGCN21 vector, in front of an *Frt-kanamycin* (*kan*)*-Frt* cassette that contains an EM7 promoter to drive *kan* transcription in bacteria.

BAC clone RP23-122D14 was acquired from BACPAC Resource Center at Children's Hospital Oakland Research Institute. This 196.9 kb BAC contains the 19.6 kb mouse *Wnt11* locus as well as 102.7 kb genomic sequence 5′ and 74.5 kb genomic sequence 3′ of *Wnt11* (Fig. 1A). To target CreER T2 into the *Wnt11* region, *CreER T2-Frt-kan-Frt* cassette was amplified using primers *Wnt11-CreER F* (GCGGTGGCCTGCAGGCGGCGGAGTTCGGTGCGGCTCCTGCAGGGTG-CGACCCCCGGGAGCGCCGGGCGCGCGCGACGATGTCCAATTTACTGA-CCGTA) and *Wnt11-CreER R* (TCGCAGATTTTGGTGGGCTCACC-CAACCTCTCCAGCTTCTCGCCCAATGGCCCATTGGAGTGAAAACGGAG-TCCTACTCTATTCCAGAAGTAGTGAGGA) to add 78 bp homology arms on each end. RP23-122D14 BAC DNA was purified and electroporated into EL250 cells, and standard BAC recombineering procedures (Lee et al., 2001) were carried out using the *Wnt11 CreER T2-Frt-kan-Frt* targeting cassette. LB agar plates containing 12.5 μg/ml chloramphenicol and 25 μg/ml kanamycin were used to select for successfully targeted clones. PCR and sequencing reactions were performed to confirm that CreER T2 was properly targeted into the *Wnt11* locus. Since EL250 cells also contain arabinose-inducible Flpe (Lee et al., 2001), the *Frt* flanked *kan* could also be deleted. The resulting *Wnt11-CreER T2* BAC DNA was purified and used for pro-nuclear injection to create *Wnt11-CreER* transgenic founders.

Syntenic analysis of the vertebrate Wnt11 genes

Genetic elements surrounding the *Wnt11* locus in mouse and its homologs in other vertebrate, namely human, chicken, zebrafish and *Xenopus* were identified using the UCSC genome browser, the zfin genome browser and the xenbase genome browser for