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Wnt5a and Wnt11 inhibit the canonical Wnt pathway and promote cardiac progenitor development via the Caspase-dependent degradation of AKT

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ABSTRACT

Wnt proteins regulate cell behavior via a canonical signaling pathway that induces β-catenin dependent transcription. It is now appreciated that Wnt/β-catenin signaling promotes the expansion of the second heart field (SHF) progenitor cells that ultimately give-rise to the majority of cardiomyocytes. However, activating β-catenin can also cause the loss of SHF progenitors, highlighting the necessity of precise control over β-catenin signaling during heart development. We recently reported that two non-canonical Wnt ligands, Wnt5a and Wnt11, act cooperatively to attenuate canonical Wnt signaling that would otherwise disrupt the SHF. While these data reveal the essential role of this anti-canonical Wnt5a/Wnt11 signaling in SHF development, the mechanisms by which these ligands inhibit the canonical Wnt pathway are unclear. Wnt11 was previously shown to inhibit β-catenin and promote cardiomyocyte maturation by activating a novel apoptosis-independent function of Caspases. Consistent with these data, we now show that Wnt5a and Wnt11 are capable of inducing Caspase activity in differentiating embryonic stem (ES) cells and that hearts from Wnt5a$^{-/-}$; Wnt11$^{-/-}$ embryos have diminished Caspase 3 (Casp3) activity. Furthermore, SHF markers are reduced in Casp3 mutant ES cells while the treatment of wild type ES cells with Caspase inhibitors blocked the ability of Wnt5a and Wnt11 to promote SHF gene expression. This finding was in agreement with our *in vivo* studies in which injecting pregnant mice with Caspase inhibitors reduced SHF marker expression in their gestating embryos. Caspase inhibition also blocked other Wnt5a/Wnt11 induced effects, including the suppression of β-catenin protein expression and activity. Interestingly, Wnt5a/Wnt11 treatment of differentiating ES cells reduced both phosphorylated and total Akt through a Caspase-dependent mechanism and phosphorylated Akt levels were increased in the hearts Caspase inhibitor treated. Surprisingly, inhibition of either Akt or PI3K in ES cells was an equally effective means of increasing SHF markers compared to treatment with Wnt5a/Wnt11. Moreover, Akt inhibition restored SHF gene expression in Casp3 mutant ES cells. Taken together, these findings suggest that Wnt5a/Wnt11 inhibit β-catenin to promote SHF development through Caspase-dependent Akt degradation.

Introduction

Congenital heart disease (CHD) affects nearly 1% of live births and is the most common class of birth defect (Tennstedt et al., 1999). The intercellular signals that coordinate heart development and how defects in these cues cause CHD have therefore become areas of intense interest within developmental biology since a better understanding of these processes may aid the discovery of new methods to detect and treat CHD. Cardiac muscle is derived from two groups of progenitor cells initially located within a region of mesoderm underlying the head folds of early embryos called the cardiac crescent (Buckingham et al., 2005; Cai et al., 2003; Kelly et al., 2001; Kelly and Buckingham, 2002; Marguerie et al., 2006; Zaffran et al., 2004). First heart field (FHF) cardiac progenitor cells occupy the lateral region of the cardiac crescent, which will come into contact at the ventral midline as the body wall closes (Buckingham et al., 2005; Zaffran et al., 2004). Once at

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the midline, FHF progenitors form a tube and differentiate rapidly to form the linear heart of the early embryo, which will later give rise to the left ventricle (Buckingham et al., 2005; Zaffran et al., 2004). Second heart field (SHF) cardiac progenitors, which are identified by their expression of the lim/homeobox transcription factor Islet1 (Isl1), occupy more medial areas within cardiac crescent that become the mesothelium dorsal to the heart tube called the dorsal mesocardium (DM) (Cai et al., 2003; Verzi et al., 2005). In contrast to FHF progenitors, SHF progenitors remain undifferentiated and expand within the pharyngeal mesenchyme and DM before migrating to the poles of heart tube to become the majority of cardiomyocytes in the remaining chambers (Cai et al., 2003; Verzi et al., 2005).

In addition to the FHF and SHF, the cardiac neural crest (CNC) plays an essential role in cardiac development. CNC cells are initially located in the dorsal neural tube but delaminate and migrate ventrally into the heart, where they form the initial septum between the aorta and pulmonary trunk and contribute smooth muscle to the proximal outflow tract and ascending aorta (Snider et al., 2007). CNC cells also invade the semilunar cushion and are essential for outflow tract (OFT) valve development even though their descendants are not maintained in the adult structures (Snider et al., 2007). SHF progenitor and CNC cells lie in close proximity to one another and communication between the two cell types is essential for the proper development of both pools of progenitors (Rentschler et al., 2010; Rochais et al., 2009; Snarr et al., 2008; Snider et al., 2007; Vincent and Buckingham, 2010). Perturbations of the SHF and CNC therefore cause a similar spectrum of OFT defects, including persistent truncus arteriosis, double outlet right ventricle, tetralogy of Fallot and ventricular septal defects (Gittenberger-de Groot et al., 2013; Keyte and Hutson, 2012; Snider et al., 2007). Moreover, recent intersectional fate-mapping experiments have identified a lineage of cells that expresses both Wnt1 and Isl1 at some time in their history (Engleka et al., 2012). Since Wnt1 is only expressed in CNC cells within the neural tube and repressed shortly after these cells delaminate and begin their migration toward the heart (Danielian et al., 1997), these data have been interpreted to mean that a subset of CNC cells express Isl1, though it is also possible that there is transient Wnt1 expression in some SHF progenitors that has yet to be detected.

Wnt proteins are secreted ligands that signal through multiple pathways to regulate critical cellular behaviors, including the determination of cell fates, the rates of cellular proliferation, survival and differentiation as well as the levels cellular motility and adhesion (Cadigan and Nusse, 1997; Niehrs and Acebron, 2012; Teo and Kahn, 2010). Effects of Wnt proteins on gene expression are most often mediated by a canonical Wnt signaling pathway that inactivates two constitutively active kinases, Glycogen synthase kinase 3α and Glycogen synthase kinase 3β (Gsk3α/β), that act in a complex with other proteins to target the unbound form of β-catenin, a cell-cell adhesion protein, for degradation (Doble and Woodgett, 2003; Wu and Pan, 2010). Therefore, in the presence of Wnt signaling, β-catenin accumulates to high levels within the cytoplasm and nucleus, where it complexes with TCF/Lef1 family transcription factors to induce target gene transcription (Angers and Moon, 2009; Eastman and Grosschedl, 1999). In addition to signaling through β-catenin, Wnt proteins can activate non-canonical effectors such as Rho-family small GTPases, Mitogen activated protein kinases (MAPK) and Protein kinase C (PKC) (Kuhl, 2002; Strutt, 2003; Tada et al., 2002). This non-canonical Wnt signaling regulates the cytoskeleton to control cellular polarity, motility and adhesion. Additionally, non-canonical Wnt signaling frequently inhibits the canonical Wnt pathway and is believed to restrict the levels and duration of canonical Wnt signaling in several contexts (Mikels and Nusse, 2006; Topol et al., 2003; Westfall et al., 2003).

The balance between canonical and non-canonical Wnt signaling is essential for the growth and differentiation of SHF cardiac progenitor cells. We as well as others have found that loss of β-catenin in both the FHF and SHF reduces the numbers of SHF progenitor cells and disrupts SHF-derived structures without affecting the FHF, suggesting that the SHF is uniquely dependent on β-catenin dependent transcription (Ai et al., 2007; Cohen et al., 2008, 2007; Klaus et al., 2007; Kwon et al., 2007; Lin et al., 2007). In contrast, expressing a cre-inducible constitutively active form of β-catenin can either increase or decrease the numbers of SHF progenitor cells depending on the cre-line used to induce its expression (Cohen et al., 2007; Kwon et al., 2007, 2009; Qyang et al., 2007). Consistent with these later data, two non-canonical Wnt proteins expressed at the anterior pole to the heart tube during SHF migration, Wnt5a and Wnt11, promote cardiogenesis in the normally non-cardiogenic posterior mesoderm of early embryos as well as differentiating stem cells by inhibiting canonical Wnt signaling (Eisenberg and Eisenberg, 1999; Koyanagi et al., 2005, 2009; Pandur et al., 2002; Schneider and Mercola, 2001; Terami et al., 2004). Yet while these data suggest that Wnt5a and Wnt11 act in early cardiac progenitors, *Wnt5a* and *Wnt11* mutations cause mild heart defects due to problems in cell–cell adhesion and cytoskeleton organization in differentiating cardiomyocytes (Nagy et al., 2010; Schleiffarth et al., 2007; Zhou et al., 2007). However, we have more recently shown that mice lacking both Wnt5a and Wnt11 die early in embryogenesis with single chambered hearts resembling those caused by SHF ablation (Cohen et al., 2012). Further analysis revealed a loss of SHF progenitor cells and an associated increase in canonical Wnt signaling within the OFT and adjacent mesenchyme of $Wnt5a^{-/-}$; $Wnt11^{-/-}$ embryos relative to controls. Treating embryoid bodies (EBs) with Wnt5a and Wnt11 also caused a synergistic increase in cardiac progenitor gene expression, an effect that is blocked by forced β-catenin activation and mimicked by canonical Wnt pathway inhibition. Together, these data indicate that Wnt5a and Wnt11 act cooperatively to restrain canonical Wnt signaling in the SHF that would otherwise disrupt cardiac progenitor development.

The effects of Wnt5a and Wnt11 on SHF progenitors may be mediated by the Caspase-dependent inhibition of the canonical Wnt pathway. Wnt11 overexpression has been shown to promote later events of cardiomyocyte differentiation, such as the expression of cardiac Troponin T and the appearance of sarcomeres, in cultures of differentiating P19 embryonic carcinoma cells by inhibiting the canonical Wnt pathway (Abdul-Ghani et al., 2011). Interestingly, these effects of Wnt11 were associated with an increase in the activity of Caspases, a large family of cysteine proteases that play critical roles in programmed cell death (Elmore, 2007; McIlwain et al., 2013), and can be blocked by the addition of Caspase inhibitors to the culture media (Abdul-Ghani et al., 2011). Importantly, the increase in Caspase activity observed with Wnt11 was not associated with an increase in the numbers of cells marked by TUNEL staining (Abdul-Ghani et al., 2011), suggesting it is unrelated to apoptosis. Caspases have also been shown to have apoptotic-independent roles in the differentiation of skeletal muscle (Fernando et al., 2002; Murray et al., 2008). All in all, these data suggest that Wnt11 promotes cardiomyocyte maturation via the Caspase-dependent inhibition of the canonical Wnt pathway. While the aforementioned study focused on later cardiac development, these data raised the possibility that the cooperative effects of Wnt5a and Wnt11 on the early development of cardiac progenitor cells are similarly mediated by non-apoptotic Caspase signaling.

The data presented in this manuscript suggest that cooperative Wnt5a/Wnt11 signaling inhibits the canonical Wnt pathway and promotes SHF development via the Caspase-dependent inhibition of Akt. Treatment of mouse ES cells derived EBs with recombinant Wnt5a and Wnt11 increased the processing of Casp3, Caspase 6 (Casp6) and Caspase 7 (Casp7) into their active forms, which correlated with increased function as evidenced by the activation of a luciferase based Casp3/7 reporter. Conversely, staining for