Wnt11-R, a protein closely related to mammalian Wnt11, is required for heart morphogenesis in *Xenopus*

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Received for publication 4 August 2004, revised 30 November 2004, accepted 2 December 2004

**Abstract**

Wnt11 is a secreted protein that signals through the non-canonical planar cell polarity pathway and is a potent modulator of cell behavior and movement. In human, mouse, and chicken, there is a single Wnt11 gene, but in zebrafish and *Xenopus*, there are two genes related to Wnt11. The originally characterized *Xenopus* Wnt11 gene is expressed during early embryonic development and has a critical role in regulation of gastrulation movements. We have identified a second *Xenopus* Wnt11-Related gene (Wnt11-R) that is expressed after gastrulation. Sequence comparison suggests that *Xenopus* Wnt11-R, not Wnt11, is the ortholog of mammalian and chicken Wnt11. *Xenopus* Wnt11-R is expressed in neural tissue, dorsal mesenchyme derived from the dermatome region of the somites, the brachial arches, and the muscle layer of the heart, similar to the expression patterns reported for mouse and chicken Wnt11. *Xenopus* Wnt11-R exhibits biological properties similar to those previously described for *Xenopus* Wnt11, in particular the ability to activate Jun-N-terminal kinase (JNK) and to induce myocardial marker expression in ventral marginal zone (VMZ) explants. Morpholino inhibition experiments demonstrate, however, that Wnt11-R is not required for cardiac differentiation, but functions in regulation of cardiac morphogenesis. Embryos with reduced Wnt11-R activity exhibit aberrant cell–cell contacts within the myocardial wall and defects in fusion of the nascent heart tube.

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**Keywords:** Wnt11; *Xenopus*; Heart development; Heart tube morphology; Cell adhesion

**Introduction**

During vertebrate development, splanchnic mesoderm symmetrically located on either side of the midline is specified to form cardiac tissue (DeRuiter et al., 1992; Garcia-Martinez and Schoenwolf, 1993; Nascone and Mercola, 1995; Sater and Jacobson, 1989; reviewed in Schoenwolf and Garcia-Martinez, 1995). During subsequent development, the paired cardiogenic tissues migrate ventrally until they fuse at the ventral midline of the embryo (reviewed by Harvey, 1999, 2002; Mohun and Sparrow, 1997; Mohun et al., 2003; Zaffran and Frasch, 2002). The heart primordia can be distinguished from the surrounding mesoderm by expression of several cardiac transcription factors including Nkx2-5, GATA4, and myocardin (Heikinheimo et al., 1994; Kelley et al., 1993; Lyons et al., 1995; Wang et al., 2001). In the *Xenopus* embryo, the cardiac progenitors begin to express sarcomeric differentiation products, including cTnl, MLC-2, and MHCα, prior to fusion of the heart patches (Drysdale et al., 1997; Raffin et al., 2000; reviewed in Mohun et al., 2003). At the early tailbud stage, the paired heart primordia fuse, first at the ventral margin to form a U-shaped incomplete tube and later at the dorsal surface to form a closed linear heart tube. After tube formation, the heart undergoes a series of morphogenetic movements to form the final three-dimensional structure consisting of the outflow tract and atrial and ventricular chambers (Harvey, 2002; Mohun et al., 2000, 2003; Zaffran and Frasch, 2002).
A subset of the Wnt family of secreted growth factors, known collectively as non-canonical Wnts, has been demonstrated to play a role in modulating morphogenesis and cell movements during development (reviewed in McEwen and Peifer, 2000; Pandur et al., 2002a; Tada et al., 2002). Wnt ligands bind to cysteine-rich frizzled and ROR receptors and LRP5/6 coreceptors (Bhanot et al., 1996; Hikasa et al., 2002; Wang et al., 2002; Wehrli et al., 2000). After ligand binding, Wnt signaling is transduced through several different pathways depending on the particular Wnt protein involved. The canonical Wnt pathway signals through dishevelled and results in the stabilization of cytoplasmic β-catenin which then forms a transcriptional complex with TCF/LEF family of HMG-box transcription factors to activate expression of downstream genes (van de Wetering et al., 1997). Alternatively, non-canonical Wnt proteins activate a signaling pathway that involves rho-GTPase, cdc42, and PKCα, and which results in activation of Jun N-terminal kinase (JNK) (Kinoshita et al., 2003; Marlow et al., 2002; Penzo-Mendez et al., 2003; Yamanaka et al., 2002). It appears that non-canonical Wnts can also signal through Rho kinase (Marlow et al., 2002) and through alterations in intracellular Ca2+ levels to bring about activation of PKC (Sheldahl et al., 1999), CAMKII, and Calcineurin (Kuhl et al., 2000). One of the non-canonical Wnt proteins, Wnt11, has been detected in the developing myocardium of mouse and chicken embryos, suggesting a role in some aspect of cardiac development (Christiansen et al., 1995; Eisenberg et al., 1997).

Mis-expression of non-canonical Wnts can cause gross malformations in embryonic development (Penzo-Mendez et al., 2003; Ungar et al., 1995). These morphological defects result from disruption of normal cell movements, cell polarity, and cell adhesion rather than from alterations in cell differentiation (Marlow et al., 2002; Moon et al., 1993; Penzo-Mendez et al., 2003; Ungar et al., 1995). In Xenopus, Wnt11 is expressed at high levels in the gastrulating embryo (Ku and Melton, 1993) but, unlike chicken and mouse (Christiansen et al., 1995; Eisenberg et al., 1997), is not expressed in the developing heart. A recent report has implicated Wnt11 activity in the pathway leading to cardiac specification (Pandur et al., 2002a,b). In this study, high doses of Wnt11 induced expression of cardiac markers such as Nkx2.5 and MHCα and beating tissue was produced. The heart inducing effect of Wnt11 requires the activation of JNK and PKC but does not require CAMKII (Pandur et al., 2002b). This inductive role for Wnt11 is very different from the previously described role for Wnt11 in regulation of gastrulation movements.

Here we present the characterization of Wnt11-R, a second Wnt11 sequence in Xenopus, that is probably the ortholog of chick and mammalian Wnt11. Xenopus Wnt11-R is expressed in the differentiating heart myocardium in a spatial and temporal expression consistent with a role in heart tube morphogenesis. We show that Wnt11-R is not required for cardiac specification or for expression of myocardial markers but is necessary for normal heart morphogenesis.

Materials and methods

Cloning of Xenopus laevis Wnt11-Related

Total RNA from stage 45 X. laevis hearts was oligo(dT)-primed and reverse transcribed for use as a template for PCR. Degenerate primers were used to amplify a 360 nt region conserved in Wnt genes using standard conditions. The forward primer (5′-CAAAAG/CGGA A/GTG/CGTG/CCAT/CGG-3′, degenerate substitutions are underlined) was designed to the peptide sequence QECCKCHG and the reverse primer (5′-A/GCAA/GCACCC/GTGA/ GTA/GAAT/CTTA/GCA-3′) was designed to the peptide sequence CKFHWCC. Amplification products were cloned and DNA sequences of representative inserts were determined to identify different Wnt sequences. Full-length Wnt11-R clones were isolated from a X. laevis whole embryo stage 42 library using the Wnt11-R PCR fragment as probe. For expression studies, the Wnt11-R coding sequence was inserted into the translation vector, pT7TS (Cleaver et al., 1996).

Embryology and microinjection

X. laevis embryos were staged according to Nieuwkoop and Faber (1994) and cultured in 0.2 × MMR. Microinjections occurred in 4% Ficoll in 0.4 × MMR and embryos were maintained in this medium for the first 12 h. Embryos were then cultured in 0.2 × MMR until harvested. For mis-expression experiments, Wnt11-R synthetic mRNA was prepared from Wnt11-R/pT7TS plasmid linearized with XbaI and transcribed with T7 RNA polymerase (Ambion). For Western analysis, injected embryos were processed at stage 12.5. For RT-PCR, VMZ and DMZ explants were dissected from injected embryos at stage 10.5 and cultured until stage 32 equivalent when they were processed. Morpholino oligos were designed to sequences in the 5′ UTR of Wnt11-R common to both pseudotetraploid copies of the gene. Wnt11-R MO1 (5′-AATCATCTTCAAAACC-CAATAACCA-3′), Wnt11-R MO2 (5′-AAACCCTAAA-CAAATAATTTTA-3′), and control MO (5′-CTTGTTACTTCTATAGCCCTATAAGGA-3′). MOs were stored in 50 mM HEPES, pH 8.0, diluted in water, and heated to 65°C for 10 min prior to injection. The Wnt11-R MO tester construction contains 350 bp of the 5′ UTR and the first three codons of Wnt11-R mRNA sequence fused to the coding region of green fluorescent protein (Wnt11-R/GFP). The tester construct was injected into the single-cell Xenopus embryo at a dose of 500 pg or coinjected with 15 ng of MO. For heart targeting, 15 ng of MO was injected at the 4 and 8 cell stage into the dorsal blastomeres in medium containing 0.4 × MMR and 4% Ficoll.
RT-PCR and Western analysis

RT-PCR primer pairs are as follows: cardiac Troponin I (Vokes and Krieg, 2002) (Tm = 63°C); MLC-2: forward: 5′-GAGGCATTCACTGTATCGA-3′, reverse: 5′-GGAATC-CAAGACTATGCATT-3′ (Tm = 60°C). Crude protein extracts from embryos and explants were prepared by homogenization and sonication in 2× SDS sample loading buffer prior to fractionation using SDS-PAGE. Western analysis and whole mount immunocytochemistry were carried out using the following primary antibodies: phospho-JNK (sc-6254, Santa Cruz Biotechnology), actin (A-4700, Sigma), diphosphorylated ERK1/2 (M-8159, Sigma), N-cadherin (610921, BD Biosciences).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out using a modification of the protocol by Harland (1991) with antisense digoxigenin-labeled probes to cardiac actin, MHCα, MLC-2, and Wnt11-R. Plasmids were linearized with Not1 and transcribed with T7 RNA polymerase using the MEGAscript kit (Ambion). For serial sections, embryos were post-fixed in 4% paraformaldehyde, embedded in Paraplast, and 10 μm transverse sections were prepared.

Results

Isolation of a second Xenopus Wnt11 sequence, Wnt11-R

In mouse and chicken embryos, the Wnt11 gene is expressed in the dorsal neural tube, dorsal dermamyotome, neural crest cells, and in the developing heart (Christiansen et al., 1995; Eisenberg et al., 1997; Kispert et al., 1996). In contrast, the Xenopus and zebrafish Wnt11 genes are expressed strongly in mesoderm tissue during gastrulation, but are not expressed in the cardiogenic region in the later stage embryo (Ku and Melton, 1993; Makita et al., 1998). In a degenerate-primer PCR survey for Wnt sequences expressed in the stage 45 X. laevis heart, we identified a Wnt11-Related sequence distinct from the previously described Xenopus Wnt11 gene. Full-length cDNA clones corresponding to the variant Wnt11 sequence (which will be referred to as Wnt11-R) were isolated from a stage 42 X. laevis embryo library and the nucleotide sequence was determined. The Xenopus Wnt11-R coding region is 1056 nt long and encodes a 352 amino acid preprotein with a calculated MW of 39.5 kDa. Alignment of the deduced Xenopus Wnt11-R protein sequence with human, chicken, zebrafish, and Xenopus Wnt11 proteins is presented in Fig. 1A. A matrix showing the percentage sequence identity between the different Wnt11 proteins (excluding the highly variable signal sequence) is shown in Fig. 1B, and we have assembled a phylogenetic tree illustrating the possible relationships of the different Wnt11 sequences (Fig. 1C). Xenopus Wnt11-R shows greater sequence identity to the human and chicken Wnt11 proteins than to the previously described Xenopus Wnt11 sequence. Xenopus Wnt11-R is 81% and 89% identical to human and chicken Wnt11 proteins, respectively, but only 64% identical to Xenopus Wnt11. In contrast, Xenopus Wnt11 shows only 64% and 66% sequence identity to the human and chicken sequences. We note also that, within the coding region, the Xenopus Wnt11-R and Wnt11 sequences are only 63% identical at the nucleotide level. This is much less than the approximately 94% nucleotide sequence identity observed for pseudoalleles in the X. laevis genome (Tonissen and Krieg, 1993), and we therefore conclude that Wnt11 and Wnt11-R represent two distinct genes. Based on sequence relationships, we propose that Wnt11-R probably represents the Xenopus ortholog of the human and chicken Wnt11 sequences. This argument is supported by the embryonic expression pattern of Xenopus Wnt11-R illustrated below.

Embryonic expression of Wnt11-R

Expression of Wnt11-R during Xenopus development was examined by in situ hybridization. Wnt11-R transcripts were first detected in the late gastrula (st. 13) in two symmetrical lines very close to the position of the neural folds (Fig. 2A). At mid-neurula (st. 22), Wnt11-R transcripts were detected along the dorsal axis in the brain, neural tube, and the somites (Fig. 2B). Transverse sections reveal that expression is confined to the extreme dorsal region of the somite (Fig. 2C). Consistent with the Wnt11 patterns in mouse and chicken, this region of expression marks the dermato-my primordia of the somite and associated mesenchymal cells. At mid-neurula, Wnt11-R transcripts were also observed in the dorsal neural tube (Fig. 2C). At later neurula stages (st. 26), expression of Wnt11-R is present in the dorsal mesenchyme extending from the otic vesicle to the tail, in the branchial arch region, and in the nervous system (Figs. 2D and E). Wnt11-R transcripts were not detected in the heart-forming region at stage 26 (Fig. 2F). At approximately stage 28, however, when the first cardiac differentiation markers are expressed, and immediately prior to fusion of the heart tube, Wnt11-R expression was visible in the heart primordia (Figs. 2G-J). In the tailbud embryo (st. 35), Wnt11-R continues to be expressed in the branchial arches, neural tube, somites, and the heart (Fig. 2K). Transverse sections at stage 35 showed Wnt11-R expression in the dorsal neural tube and in the dorsal and lateral boundaries of the somites (Fig. 2L). Expression was also seen in mesenchymal cells that populate the dorsal fin. Sections through the stage 34 heart indicated that Wnt11-R was expressed in a subset of the myocardium, in particular the anterior myocardium of the heart tube comprising the outflow tract (Fig. 2M) and
the ventricle (Fig. 2N). Only weak staining was seen in the posterior heart tube representing the future atrial myocardium (Fig. 2O). By the tadpole stage (st. 45), Wnt11-R is expressed throughout the myocardium but more strongly in the ventricular tissue (Fig. 2Q).

Mis-expression of Wnt11-R activates JNK and induces cardiac marker expression

We have carried out a series of experiments to determine whether Wnt11-R shares biological activities with Wnt11.
First, we show that Wnt11-R does not form secondary axes when mis-expressed in the *Xenopus* embryo. Previous studies have shown that Wnt proteins signaling through the beta-catenin pathway generate secondary axes with high frequency when expressed in ventral blastomeres (Pandur et al., 2002a). For example, we were able to demonstrate that expression of the canonical Wnt8a sequence was highly efficient at producing duplicated axes (Fig. 3B). However, secondary axes were not produced by similar doses of Wnt11-R (Fig. 3C). Second, previous studies have shown that ectopic expression of Wnt11 in the *Xenopus* embryo causes severe disruption of normal gastrulation movements (Penzo-Mendez et al., 2003; Tada and Smith, 2000) and we have confirmed this result for Wnt11-R. As shown in Table I,
synthetic Wnt11-R mRNA microinjected into single cell stage embryos resulted in defective gastrulation at doses from 100 pg to 1 ng. Even at 100 pg, all Wnt11-R-injected embryos showed gastrulation defects including incomplete closure of the blastopore and neural tube (Fig. 3E). These defects were not observed in control embryos microinjected with 500 pg of GFP mRNA (Fig. 3D).

Third, we assayed whether Wnt11-R could activate JNK and induce cardiac marker expression, as previously demonstrated for Wnt11 (Pandur et al., 2002b). Embryos were injected with different amounts of synthetic Wnt11-R mRNA at the one cell stage and then protein extracts from explants of gastrula stage embryos (st. 12.5) were assayed for JNK activation by Western blot using a phospho-JNK specific antibody (Fig. 3F). This assay showed a dose-dependent increase in JNK activation with increasing levels of Wnt11-R. The treatment did not result in an increase in levels of diphosphorylated ERK1/2, which is not activated by Wnt11 signaling (Fig. 3F). The Wnt11-R induced activation of JNK in VMZ explants could be blocked by the JNK inhibitor SP600125 (Fig. 3G). RNA extracts from VMZ explants of Wnt11-R injected embryos (st. 32 equivalent) were tested for the presence of cardiac marker transcripts using RT-PCR (Fig. 3 H). Two myocardial specific markers MLC-2 and cTnI were detected in VMZ explants from embryos injected with high levels (1 ng) of Wnt11-R mRNA but not at the lower doses that were sufficient to disrupt gastrulation movements. Overall, these experiments indicate that Wnt11-R signals through the non-canonical Wnt pathway and exhibits biological activities equivalent to Wnt11.

### Table 1

Embryonic over-expression of Wnt11-R causes morphological defects

<table>
<thead>
<tr>
<th>Injected mRNA</th>
<th>Morphological defects (assayed a stage 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ng EGFP</td>
<td>2% (n = 56)</td>
</tr>
<tr>
<td>100 pg Wnt11-R</td>
<td>96% (n = 75)</td>
</tr>
<tr>
<td>250 pg Wnt11-R</td>
<td>100% (n = 36)</td>
</tr>
<tr>
<td>500 pg Wnt11-R</td>
<td>98% (n = 58)</td>
</tr>
<tr>
<td>1 ng Wnt11-R</td>
<td>100% (n = 89)</td>
</tr>
</tbody>
</table>

To investigate a possible role for Wnt11-R in heart development, we utilized a morpholino (MO) antisense strategy to translationally inhibit the expression of Wnt11-R. Two morpholinos, MO1 and MO2, were designed, each corresponding to regions showing 100% sequence identity...
between transcripts from the pseudoallelic *laevis* Wnt11-R genes. The ability of these MOs to inhibit translation of Wnt11-R mRNA in the embryo was tested using a synthetic mRNA consisting of the 5' UTR and first 3 codons of Wnt11-R fused to the coding region of EGFP (Fig. 4A). Injection of 500 pg of this synthetic mRNA into Xenopus embryos produced bright fluorescence due to translation of EGFP protein (Fig. 4B). Coinjection of the tester mRNA with 15 ng of either MO1 or MO2 showed that only the MO overlapping the initiation codon (MO1) was able to inhibit translation of EGFP and block fluorescence (Fig. 4D). MO2 produced no detectable reduction in fluorescence (data not shown). This assay appears to be a useful test for inhibitor activity, since subsequent experiments showed that MO2 had no detectable effect on Wnt11-R function in the embryo (data not shown). The inhibition of EGFP translation by MO1 was confirmed by protein blotting using antibodies directed against GFP (Fig. 4E). As expected, a control MO containing 11 base changes relative to MO1 had no detectable effect on EGFP expression (data not shown).

To determine the developmental consequences of inhibition of Wnt11-R expression in the heart, 15 ng of MO1 was injected into one or both of the D2 blastomeres of the 8-cell embryo. Fate mapping shows that these cells contribute to heart and somitic tissues (Dale and Slack, 1987; Moody, 1987). The un.injected side of the embryo serves as a stage matched control. In initial experiments, MO1-injected embryos were allowed to develop until early tailbud (st. 28) when they were assayed for expression of cardiac differentiation markers. As shown in Fig. 4G, inhibition of Wnt11-R expression had no detectable effect on expression of MHCα, which is an early and very strong marker of myocardial differentiation (Logan and Mohun, 1993). Similarly, expression of the cardiac marker MLC-2 was normal in MO1-injected embryos (Table 2) and no effects on cardiac gene expression were observed when embryos were cultured until the tailbud stage (Table 2). Assays with specific cardiac probes cannot exclude the possibility that Wnt11-R is important for regulation of an unspecified gene that is essential for normal myocardial differentiation. To test for consequences later in development, MO1 was injected bilaterally and embryos were allowed to develop until the tadpole stage (st. 45). In 100% of cases, double-side-injected embryos had beating hearts (Table 2), indicat-

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**Fig. 4.** Wnt11-R antisense morpholino oligomer inhibits translation from Wnt11-R-GFP fusion transcripts. (A) Diagram of the Wnt11-R-GFP fusion (tester) mRNA, indicating binding locations for antisense MO1 and MO2. The ability of MOs to inhibit translation from mRNAs containing Wnt11-R sequences was assayed by coinjecting 500 pg of synthetic tester mRNA with 15 ng of Wnt11-R MO1 into fertilized eggs. (B–D) At about stage 13, embryos were viewed under UV light or standard illumination. Uninjected embryos (B) serve as negative control and do not glow. Embryos injected with tester mRNA (C) fluoresce, confirming the translation of GFP protein. Embryos coinjected with MO1 plus tester mRNA (D) show strongly reduced fluorescence, indicating effective inhibition of translation. The second Wnt11-R antisense MO, MO2, showed no inhibition of GFP expression using this assay (data not shown). (E) Immunoblot detection of GFP protein in whole embryo extracts from uninjected embryos, tester mRNA-injected embryos, and embryos coinjected with tester mRNA and 15 ng of MO1. GFP protein is detected in the tester sample, but is not detectable when translation is inhibited by MO1. Ponceau S staining indicates equal loading of sample. (F) Embryo injected with 15 ng of control MO (contMO) assayed at st. 28 by in situ hybridization for MHCα, showing normal cardiac differentiation. (G) Embryo injected with 15 ng of MO1 also shows normal appearance of differentiation markers when assayed for MHCα transcripts.
Table 2
Expression of cardiac differentiation markers and cardiac beating is normal in Wnt11-R MO-treated embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Heart patch symmetry (assayed at stage 28) MHCα</th>
<th>MLC-2</th>
<th>MHCα expression (assayed at stage 35)</th>
<th>Beating hearts (stage 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>97% (31)</td>
<td>93% (31)</td>
<td>100% (34)</td>
<td>100% (39)</td>
</tr>
<tr>
<td>Control MO</td>
<td>97% (35)</td>
<td>96% (24)</td>
<td>100% (83)</td>
<td>100% (17)</td>
</tr>
<tr>
<td>Wnt11-R MO1</td>
<td>92% (39)</td>
<td>93% (28)</td>
<td>100% (70)</td>
<td>100% (87)</td>
</tr>
</tbody>
</table>

ing that all genes required for cardiac contractility were expressed in Wnt11-R-depleted embryos.

Inhibition of Wnt11-R function using morpholino oligomers causes defects in heart morphogenesis

We examined the heart region of Wnt11-R-depleted embryos to detect possible alterations in cell behavior or cell movement. During early tailbud stages, double-sided MO1-injected embryos formed normal patches of precardiac tissue that migrated to the ventral midline (Figs. 5A–D). The first alterations in cardiac morphology became evident during the process of heart tube formation (Figs. 5E–K). At stage 34, transverse sections through MO-treated embryos showed that, although the cardiac primordia migrated normally and came to abut at the ventral midline, the heart primordia did not fuse properly. This resulted in a subset of hearts showing that all gene required for cardiac contractility were expressed in Wnt11-R-depleted embryos.

Non-canonical Wnt signaling is mediated, in part, through activation of the JNK pathway (reviewed in Pandur et al., 2002a; Tada et al., 2002), and we have demonstrated that Wnt11-R is capable of JNK activation in embryonic tissue (Fig. 3F). Phosphorylation of JNK in the cytoplasm results in translocation of JNK protein to the nucleus (Reviewed in Weston and Davis, 2002). Whole mount immunostaining shows that the phospho-JNK protein was broadly distributed in the early tailbud embryo, including the precardiac region (Fig. 6A). To determine if activated JNK is present in the myocardium, we examined early heart tube stage embryos (st. 30) for nuclear localized JNK staining. Phosphorylated JNK could be detected using the anti-phospho-JNK antibody, SC-6254, within the myocardial tissue layer in sectioned embryos (Figs. 6B and C). Staining was diffuse, but concentrated in the nucleus and perinuclear region of the myocardial cells, as marked by propidium iodide staining (Fig. 6D).

The presence of activated JNK in the myocardium is consistent with a role for JNK in mediating Wnt11-R signaling during heart tube formation. We therefore tested whether inhibition of JNK activation, using the chemical inhibitor SP600125, would replicate the cardiac phenotypes of Wnt11-R MO treatment. Incubation of Xenopus embryos with 20 μM SP600125, starting at stage 26 (approximately 2 h prior to the onset of Wnt11-R transcription in the heart) until stage 33, had no effect on embryo morphology, and myocardial marker expression occurred at the normal time (Figs. 6E and G). Transverse sections through treated embryos, however, revealed abnormalities in heart morphology that were apparently identical to those in Wnt11-R morpholino-treated embryos. The hearts of SP600125-treated embryos exhibited defects in heart tube fusion at the ventral midline and also an increase in myocardial area (Fig. 6H). Quantitation showed that the myocardial area was increased 19% relative to controls. Another regulatory protein known to be involved in transduction of Wnt signaling pathways is Ca(2+)/calmodulin-dependent protein kinase II, CaMKII (Kuhl et al., 2000), and previous studies have utilized the CaMKII inhibitor KN-93 in Xenopus embryos (Pandur et al., 2002b; Wu and Cline, 1998). When we treated embryos with 20 μM KN-93, under identical conditions to those used for SP600125, we observed no defects in normal heart tube fusion, and no significant alteration in myo-
cardiac area relative to controls (data not shown). These results suggest that the JNK pathway, but not the CaMKII pathway, is involved in transduction of Wnt11-R signaling in the developing heart.

Three different mechanisms might account for the increase in myocardial area observed in Wnt11-R-morpholino and JNK inhibitor-treated embryos; increased numbers of cells, increased size of cells, or increased space between the cells. A combination of more than one mechanism could also be at work. We determined the number of cells in the myocardial wall of double-sided MO1-treated embryos and stage-matched untreated control embryos. Quantitation of cell nuclei number using DAPI visualization (Fig. 5P) showed no statistical difference in cell number in the MO treated and control myocardia (Table 3). Similarly, no significant difference was observed when cell numbers were determined for the myocardial layer of SP600125-treated embryos (Table 3).
We next used transmission electron microscopy (TEM) to take a closer look at myocardial cells. Sections through a control heart at stage 34 showed that the myocardium consisted of a single layer of columnar cells (Fig. 6I). In most cases, the nuclei were located closer to the outer surface of the heart tube. Each myocardial cell was closely juxtaposed to its neighbors with little extracellular space visible at the margin between cells. Measurements from the TEM images showed that the average area of a myocardial cell in Wnt11-R morpholino and JNK inhibitor-treated embryos (Figs. 6J and L) was not significantly different from that of sibling control embryos (Table 3). However, Wnt11-R MO and JNK inhibitor-treated embryos showed an increase in the extracellular space between neighboring cells (marked in yellow in Figs. 6I–L) possibly due to decreased adhesion between cells. Quantitation indicated that the proportion of the total myocardial area represented by open extracellular space was 4-fold greater in MO-treated embryos than in controls (16% vs. 4%, respectively). Similarly, inhibition of JNK activation increased the

Table 3
Increased myocardial area in Wnt11-R MO-treated and JNK inhibitor-treated embryos

<table>
<thead>
<tr>
<th>Morpholino treatment</th>
<th>No. of nuclei in myocardium (assayed stage 34)</th>
<th>Area of myocardium (arbitrary units)</th>
<th>Cells/unit area</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control MO</td>
<td>59.4 ± 4.8 (8)</td>
<td>78.1 ± 12.4 (8)</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Wnt11-R MO1</td>
<td>59.1 ± 9.3 (7)</td>
<td>98.4 ± 20.6 (7)</td>
<td>0.60</td>
<td>0.017</td>
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<table>
<thead>
<tr>
<th>Inhibitor treatment</th>
<th>No. of nuclei in myocardium (assayed stage 32)</th>
<th>Area of myocardium (arbitrary units)</th>
<th>Cells/unit area</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (carrier)</td>
<td>43.1 ± 4.6 (8)</td>
<td>63.1 ± 10.5 (8)</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>DMSO + SP600125 (20 µg/ml)</td>
<td>43.0 ± 6.7 (11)</td>
<td>75.0 ± 14.3 (11)</td>
<td>0.57</td>
<td>0.026</td>
</tr>
</tbody>
</table>

We next used transmission electron microscopy (TEM) to take a closer look at myocardial cells. Sections through a control heart at stage 34 showed that the myocardium consisted of a single layer of columnar cells (Fig. 6I). In most cases, the nuclei were located closer to the outer surface of the heart tube. Each myocardial cell was closely juxtaposed to its neighbors with little extracellular space visible at the margin between cells. Measurements from the TEM images showed that the average area of a myocardial cell in Wnt11-R morpholino and JNK inhibitor-treated embryos (Figs. 6J and L) was not significantly different from that of sibling control embryos (Table 3). However, Wnt11-R MO and JNK inhibitor-treated embryos showed an increase in the extracellular space between neighboring cells (marked in yellow in Figs. 6I–L) possibly due to decreased adhesion between cells. Quantitation indicated that the proportion of the total myocardial area represented by open extracellular space was 4-fold greater in MO-treated embryos than in controls (16% vs. 4%, respectively). Similarly, inhibition of JNK activation increased the
extracellular space to 13% of the total myocardial area. We suggest that the observed increase in extracellular space in MO-treated or inhibitor-treated embryos relative to controls is a major factor contributing to increase in the area of the myocardial tissue layer. However, extracellular space alone is not sufficient to account for the entire difference in area (approximately 25% greater than controls), suggesting that other factors, not readily detectable using TEM, must also contribute to the knockdown phenotype. One possibility is that the reduced adhesion leads to slippage of cells out of a strictly columnar arrangement, leading to increased width of the myocardial layer.

Discussion

Vertebrate Wnt11 and Wnt11-Related genes

During development of *Xenopus* and zebrafish, a Wnt11 gene is expressed in the involuting mesoderm of the gastrula stage embryo (Ku and Melton, 1993; Makita et al., 1998). Later during development, this gene is expressed in the branchial arch region and the somite, but not in the heart. Dominant-negative inhibition studies in *Xenopus* and mutant analysis in zebrafish indicate that this Wnt11 gene plays an important role in regulation of gastrulation movements (Heisenberg et al., 2000; Tada and Smith, 2000; Ulrich et al., 2003). In contrast, the Wnt11 genes identified in chicken and mouse are not expressed in the gastrulating mesoderm (Christiansen et al., 1995; Eisenberg et al., 1997; Kispert et al., 1996). Transcripts of the chicken and mouse Wnt11 genes are observed in the branchial arch region, the dermamyotome and adjacent dorsal mesenchyme of the somites, and in the developing heart. We have identified Wnt11-R, a second *Xenopus* Wnt11 gene that, based on sequence comparison and embryonic expression pattern, is likely to represent the ortholog of the single Wnt11 gene reported in chicken and mouse. A second Wnt11-Related sequence is also present in zebrafish (accession number AAH66498 and Fig. 1A), and while no expression information for this gene is currently available, we predict that it will exhibit a pattern similar to that of Wnt11-R. It seems likely that the multiple Wnt11 genes in fish and frogs resulted from an ancient gene duplication event, but that the second copy has been lost in birds and mammals.

Experiments using *Xenopus* embryos show that either mis-expression or inhibition of Wnt11 activity causes severe alterations in cell movements resulting in defective gastrulation (Marlow et al., 2002; Penzo-Mendez et al., 2003). In addition, high level expression of Wnt11 can cause activation of JNK and induce expression of cardiac differentiation markers in embryonic explants (Pandur et al., 2002a,b). Since *Xenopus* Wnt11 and Wnt11-R are clearly closely related sequences, we have carried out tests for biological activity and demonstrated that mis-expression of Wnt11-R also causes severe gastrulation defects (Fig. 3E) and leads to activation of JNK and induction of heart markers (Figs. 3F and H). We therefore conclude that Wnt11 and Wnt11-R share at least some of the same biological activities.

Wnt11-R is required for heart tube morphogenesis

In *Drosophila*, the planar cell polarity pathway (PCP) plays an essential role in the regulation of embryonic morphogenesis (reviewed in Fanto and McNeill, 2004). In vertebrates, the PCP pathway is stimulated by non-canonical Wnt ligands, particularly Wnt5A and Wnt11, and also functions to regulate cell behavior and movement (Tada et al., 2002; Wallingford et al., 2002). To assess whether Wnt11-R is involved in regulation of cell movement and adhesion during heart development, we downregulated Wnt11-R activity using a MO approach. First, we observed that inhibition of Wnt11-R function has no discernible effect on the timing or pattern of expression of myocardial marker genes. This is completely consistent with expectations, since Wnt11-R is not normally expressed in heart tissue until the time that cardiac markers are expressed (Figs. 2G–J). Second, we showed that although the initial stages of migration of the cardiac primordia to the ventral midline progressed normally, fusion of the primordia to form the primitive heart tube was disrupted. The most common defect, observed in almost 60% of cases, was a thickening of the myocardial wall and an accumulation of disorganized cells at the site where fusion would normally occur (Fig. 5J). In the most extreme cases, the individual heart primordia form two adjacent U-shaped tubes (Fig. 5K) and a small proportion of hearts (about 10%) went on to exhibit partial cardia bifida (Figs. 5M and N). The thickened myocardial wall that was evident in heart tube stage embryos was undetectable in later stage hearts (data not shown). This loss of phenotype could be due to degradation of the morpholino inhibitor and restoration of Wnt11-R protein levels or, alternatively, another non-canonical Wnt protein may become expressed in the heart and substitute for Wnt11-R activity. Interestingly, defective cardiac morphology is also observed in the Loop-tail mouse mutant (Lpp1) (Henderson et al., 2001; Murdoch et al., 2001). Lpp1 is related to *Drosophila* Strabismus, a factor in the PCP signaling pathway (reviewed in Heisenberg and Tada, 2002). Although the precise cause of the morphological flaws in Lpp1 mutant hearts is not clear, it is tempting to speculate that both Lpp1 and Wnt11-R defects are due disruption of the PCP pathway within the myocardium.

Wnt11 signaling through JNK functions to regulate embryonic morphogenesis, particularly in the context of convergent extension movements during gastrulation (Yamanaka et al., 2002). However, apart from gastrulation, there are no other examples of JNK-dependent morphogenesis. The experimental results presented in this paper provide three lines of evidence suggesting a role for JNK-mediated Wnt11-R signaling in regulation of cardiac morphogenesis. First, expression of Wnt11-R in *Xenopus*
embryonic tissue can induce JNK phosphorylation (Fig. 3F). Second, Wnt11-R is expressed in the heart at the time that activated JNK is present (Figs. 6C–E), consistent with a role for Wnt11-R in activation of JNK. Third, depletion of Wnt11-R by MO treatment generates phenotypes indistinguishable from those obtained by inhibiting JNK activation (Figs. 5J and 6H). We interpret these results to suggest that Wnt11-R is signaling through a currently unidentified Wnt receptor to activate JNK and regulate subsequent myocardial tissue movements. It is interesting to note that the myocardial cell layer, which is expressing Wnt11, is also the cell layer affected by Wnt11 depletion. This observation suggests that Wnt signaling is acting cell autonomously to regulate cardiac morphology, as previously proposed for regulation of gastrulation movements (Tada and Smith, 2000).

To explore possible mechanisms underlying the defects in cardiac development, we used electron microscopy to examine the structure of the myocardial wall in Wnt11-R MO and JNK inhibitor-treated embryos. Both of these treatments cause the area of myocardial tissue to be increased relative to control embryos without any detectable increase in cell number (Table 3). Our TEM studies revealed a reduction of close contact between individual cardiac cells in treated embryos, associated with a significant increase in extracellular space (approximately 4-fold over controls). These observations might be explained by reduced cell adhesion between individual cardiomyocytes comprising the wall of the heart tube. It is possible that reduced adhesion between myocardial cells allows them to slip out of the regular columnar arrangement, leading to an increase in the overall thickness of the myocardial wall (Figs. 6K and M). A role for Wnt11-R in regulating cell adhesion and cell contact is consistent with previous studies of the mouse Wnt5A gene. Knockout of Wnt5A results in ventricular septal defects (Oishi et al., 2003; Yamaguchi et al., 1999) and studies using cardiomyocytes in culture showed that increased expression of Wnt5A enhanced stability of N-cadherin protein at sites of adhesion without influencing transcription of the N-cadherin gene (Toyofuku et al., 2000). N-cadherin does not appear to underlie the effects observed in Wnt11-R-deficient Xenopus embryos, however, since we were unable to detect any alteration in N-cadherin protein levels relative to controls (data not shown).

Does Wnt11 function as a cardiac inducer?

The blastopore lip region of the frog embryo, where Wnt11 is expressed, is also the region involved in induction of the cardiac lineage (Nascone and Mercola, 1995; Sater and Jacobson, 1989). Similarly, in the chick embryo, Wnt11 is reported to be expressed in the cardiac crescent (Eisenberg et al., 1997), although subsequent studies of chick have failed to detect Wnt11 expression in the cardiogenic region (Chapman et al., 2004). Based initially on these observations of Wnt11 expression in precardiac tissues, it has been proposed that Wnt11 plays a central role in the specification of the heart lineage (Pandur et al., 2002b), and over-expression experiments using Xenopus tissue explants showed that Wnt11 can induce expression of cardiac markers. We have confirmed these results by showing that over-expression of Wnt11-R also induces expression of cardiac genes (Fig. 3H). We note, however, that Wnt11-R is not expressed in cardiac tissues until about the time of myocardial differentiation (Fig. 2) and therefore cannot normally play a role in induction of the cardiac lineage. In the frog embryo, expression of dominant-negative Wnt11 disrupts normal gastrulation movements (Tada and Smith, 2000) and zebrafish silberblick (Wnt11) mutants show defects in gastrulation (Heisenberg et al., 2000; Ulrich et al., 2003). Significantly, no cardiac defects are apparent in silberblick mutants (Heisenberg and Nusslein-Volhard, 1997), indicating that Wnt11 is not essential for specification of the cardiac lineage in zebrafish.

Taking the available information into account, we wish to suggest that induction of cardiac markers by high levels of Wnt11 and Wnt11-R may be an artifact of the over-expression assay. This proposal is based on the observation that high level expression of Wnt proteins normally involved in non-canonical signaling can inhibit the canonical Wnt signaling pathway (Topol et al., 2003; Weidinger and Moon, 2003; Yan et al., 2001). Indeed, this has been demonstrated specifically for Wnt11 (Maye et al., 2004). Previous studies in avian and frog embryos have shown that disruption of canonical Wnt signaling, using the inhibitory proteins dickkopf or crescent, results in activation of the cardiac differentiation pathway and ectopic expression of myocardial markers (Marvin et al., 2001; Schneider and Mercola, 2001). We suggest that high level expression of either Wnt11 or Wnt11-R in ventral marginal zone explants is approximately equivalent to expression of the Wnt inhibitors dkk or crescent and is sufficient to inhibit canonical Wnt signaling and to activate heart marker expression.

The results presented in this report suggest that Wnt11-R functions to control cell behavior during morphological movements. In particular, evidence has been presented showing that the disruption of cardiac morphology observed in Wnt11-R-deficient embryos is associated with changes in adhesion properties of cells in the myocardial wall. At its most extreme, these alterations in cell behavior generate a cardiabifida phenotype (Figs. 5M and N). At present, however, it is not known which genes are regulated in response to Wnt11-R signaling or which specific proteins mediate Wnt11-R-regulated cell adhesion. Loss of function studies for the single Wnt11 gene in the mouse genome should help to resolve the role of Wnt11 during cardiac development.

Acknowledgments

We wish to thank Peggy McCuskey for preparation of the Electron Microscope images. P.A.K. is the Allan C. Hudson...
References


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