**ABSTRACT**

Wnt signalling regulates cardiogenesis during specification of heart tissue and the morphogenetic movements necessary to form the linear heart. Wnt11-mediated non-canonical signalling promotes early cardiac development whilst Wnt11-R, which is expressed later, also signals through the non-canonical pathway to promote heart development. It is unclear which Frizzled proteins mediate these interactions. Frizzled-7 (fzd7) is expressed during gastrulation in the mesodermal cells fated to become heart, and then in the primary heart field. This expression is complementary to the expression of wnt11 and wnt11-R. We further show co-localisation of fzd7 with other early- and late-heart-specific markers using double in situ hybridisation. We have used loss of function analysis to determine the role of fzd7 during heart development. Morpholino antisense oligonucleotide-mediated knockdown of Fzd7 results in effects on heart development, similar to that caused by Wnt11 loss of function. Surprisingly, overexpression of dominant-negative Fzd7 cysteine rich domain (Fzd7 CRD) results in a cardia bifida phenotype, similar to the loss of wnt11-R phenotype. Overexpression of Fzd7 and activation of non-canonical wnt signalling can rescue the effect of Fzd7 CRD. We propose that Fzd7 has an important role during Xenopus heart development.

**KEY WORDS:** *Xenopus laevis*, Cardiogenesis, Wnt signalling, Fzd7

**RESULTS**

**fzd7 expression overlaps with early heart markers**

Expression pattern analysis shows *Xenopus fzd7* is expressed in the heart-forming regions throughout development (Wheeler and Hopfler, 1999). At stage 10.5 fzd7 is expressed in the dorsal mesoderm from which cardiac tissue originates (Wheeler and Hopfler, 1999) (Fig. 1A). As development progresses, fzd7 expression at stage 25 is maintained in the presumptive cardiac mesoderm as it migrates dorso-laterally to the ventral midline (Fig. 1C-Cii). By stage 29, fzd7 is expressed throughout the cardiac crescent in the cardiac mesoderm (Fig. 1E,Ei). fzd7 expression correlates with that of wnt11 (Fig. 1B, stage 10.5) where expression of both genes seem to be complementary in the presumptive heart region in the dorsal side of the embryo. fzd7 expression also correlates to that of wnt11-R (Fig. 1D-Dii,F,Fii, stages 25 and 29, respectively) where it is expressed in the anterior endoderm at stage 25 when fzd7 is expressed in the heart field. By stage 29, the expression of fzd7 and wnt11-R overlaps (Fig. 1E-Fii). As the heart processes underlying heart development and morphogenesis are important for understanding congenital heart disease.

Heart formation is controlled by many signalling pathways including wnt signalling. *Wnt6, wnt11*, and *wnt11-R* have all been implicated in *Xenopus* heart development (Garriock et al., 2005; Gessert et al., 2008; Lavery et al., 2008a; Pandur et al., 2002). Wnt antagonists such as Dickkopf-1, Crescent and Sfrp1 have also been reported to control early heart formation (David et al., 2008; Foley and Mercola, 2005; Gibb et al., 2013; Marvin et al., 2001; Schneider and Mercola, 2001). Little is known however about which Frizzled proteins mediate these signals. Frizzled-7 (fzd7) has been well characterised in *Xenopus laevis* and other species. It has been shown to be involved in numerous developmental processes as well as being shown to be active in several forms of cancer (Huang and Klein, 2004; Liu et al., 2016; Schiffgens et al., 2016; Xu et al., 2016). Fzd7 has been demonstrated to interact with several wnts including Wnt5a (animal cap elongation assays), Wnt6 (in somite development), Wnt8 (co-immunoprecipitation assays, *Xenopus* axis duplication) and Wnt11 (gastrulation movements, neural crest development) (Hsieh et al., 1999; Linker et al., 2005; Medina et al., 2000; Medina and Steinbeisser, 2000; Umbhauser et al., 2000; Witzel et al., 2006). It has also been shown to genetically interact with the coreceptors ror2 and ryk (Hikasa et al., 2002; Kim et al., 2008). *Xenopus fzd7* has been implicated in gastrulation movements, tissue separation, and neural crest induction (Abu-Elmagd et al., 2006; Djiane et al., 2000; Wheeler et al., 2000; Winklbauer et al., 2001). We have previously shown *fzd7* to be expressed in the cardiac region throughout development (Wheeler and Hopfler, 1999). It has also been shown that specific depletion of fzd7 function in *Xenopus* foregut leads to impaired cardiac morphogenesis, but has no effect on heart specification (Zhang et al., 2013). Here, we further characterise its expression relative to known heart markers, and then use whole-embryo experiments to show that *fzd7* is required for heart formation during early embryonic development.

**INTRODUCTION**

During embryogenesis, the heart is one of the first organs to form. Development of the heart includes specification of cardiac progenitors and formation of the linear heart tube by cell migration and morphogenetic movements (Mohun et al., 2000). In *Xenopus*, the heart begins to form during early gastrula stages when the cardiac progenitors arise in the dorsolateral mesoderm. Cell movements during gastrulation result in the dorso-anterior translocation of these regions and subsequent ventral migration during neurulation. The heart progenitors, which comprise cells fated to become primary or secondary heart field, form a linear heart tube at the ventral midline before looping and remodelling to form the beating heart (Kriegmair et al., 2013). Understanding the
fzd7 expression at stage 25. Complementary in the presumptive heart region. (C-Cii, D-Dii) wnt11 expression detected at the dorsal side of the embryo and appear and wnt11 (Cii, Dii). (E,Ei, F,Fi) Stage 29 embryos with wnt11-R (Fig. 2B-Bii), tmnic (Fig. 2C-Cii) and gata6 (Fig. 2D-Dii) in the forming heart. Interestingly, none of these markers are seen in the pericardium except for fzd7 (Fig. 2Aii,Bii, Cii, Dii).

fzd7 is required for heart induction or specification

Microinjection into Xenopus embryo dorsal blastomeres at the 4- or 8-cell stage targets prospective mesoderm including cardiac tissue. In order to test the role of fzd7 in heart development, we inhibited its function by injecting either fzd7 morpholino (fzd7 MO) or its dominant-negative form expressing only the extracellular domain (cysteine rich domain, fzd7 CRD), which would disrupt fzd7 mediated signalling (Abu-Elmagd et al., 2006).

Microinjection of fzd7 MO into the dorsal blastomeres of 4- or 8-cell embryos leads to a reduction of both early cardiac marker nks2-5 (Fig. 3B-Bii) and later cardiac marker tmnic expression (Fig. 3E-Eii). Adding increasing amounts of fzd7 MO leads to a progressively more severe phenotype with a greater number of embryos affected (Fig. 3C). In situ hybridisation for nks2-5 and tmnic show embryos with mild convergent extension phenotypes (Fig. 3B,E), but a severe decrease in cardiac gene expression (Fig. 3Bi,Ei) while control morpholino (CMO) show normal heart (Fig. 3A,Ai,D,Di). Some embryos also showed anterior defects (data not shown). Sections through the cardiac region showed not only a decrease of nks2-5 and tmnic expression, but an absence of recognisable heart structures (Fig. 3Bi,Ei) compared to CMO (Fig. 3Aii,Dii). The number of embryos injected with fzd7 MO which showed heart and/or convergent extension and anterior defects are shown in Table S1.

Overexpression of fzd7 full-length (fzd7 FL) results in severe convergent extension defects, but no cardiac phenotype (Fig. S1A,B). Knockdown with fzd7 MO can also cause a mild convergent extension phenotype and anterior defects (Abu-Elmagd et al., 2006). In order to test whether this cardiac effect is specific to fzd7, we rescued the fzd7 MO cardiac phenotype with fzd7 FL that has been mutated to not bind the fzd7 MO (fzd7 SDM, as described in Abu-Elmagd et al., 2006). Titrating increasing amounts of fzd7 SDM capped RNA from 250 pg to 1 ng results in a modest rescue of the cardiac phenotype (Fig. 3F,Fi; Table S2), thus showing that fzd7 is required for normal heart development.

Interestingly, injecting fzd7 FL at 8-cell stage embryos shows detectable expression of tmnic and nks2-5, despite some of these embryos showing severe convergent extension movements phenotype (head arrows in Fig. S1A,B). This leads to the suggestion that heart phenotypes are not necessarily due to convergent extension secondary effects.

fzd7 CRD mimics wnt11-R morpholino cardiogenesis phenotype and is required for non-canonical signalling

To further look at the effect of inhibiting fzd7 function, we took a dominant-negative approach using fzd7 CRD. Surprisingly, this did not give a similar result to the MO knockdown. Instead, increasing amounts of fzd7 CRD results in a dose-dependent increase in frequency and severity of cardiac bifida. This was very similar to the phenotype seen for wnt-11R knockdown (Garriock et al., 2005). Embryos with very mild convergent extension movement defects displayed a severe cardiac bifida phenotype as shown by tmnic expression.
Control embryos showed normal expression of *tnnic* (Fig. 4A-Aii) and *nkx2-5* (Fig. 4F,Fi). These results suggest that the cardiabifida phenotype is not a secondary effect of the convergent extension defect. Overexpression of *fzd7* FL gives a severe convergent extension phenotype but no cardiac phenotype (Fig. S1A,B). Embryos with cardiabifida were unable to recover and form a normal heart when incubated up to stage 41 (n=23, data not shown).

**DISCUSSION**

Wnt signalling through the canonical and non-canonical pathways has been implicated in many aspects of heart development (Gessert and Kuhl, 2010; Ruiz-Villalba et al., 2016). How the wnt signals that arise from both non-cardiogenic and cardiogenic tissue are integrated into heart development is less well understood. Frizzled proteins are only a part of the increasingly complicated wnt-receptor complex found at the cell membrane, which can also include Lrp5/6, Ror2, Ryk and Kremen (Bryja et al., 2009; Korol et al., 2008; Mazzotta et al., 2016; van Wijk et al., 2009); however, Frizzled proteins are critical components of the Wnt receptor complex and so understanding their role in heart development is necessary to fully understand the
signalling involved. We have previously shown that fzd7 is expressed throughout heart development, and in this study, we show that it is functionally required in both early and late heart development.

Morpholino knockdown of fzd7 leads to effects on heart development, including in some cases a complete loss of heart (Fig. 3). Overexpression of fzd7 gives rise to convergent extension defects as previously reported (Abu-Elmagd et al., 2006; Sumanas and Ekker, 2001; Winklbauer et al., 2001), but does not affect heart development. We can rescue the fzd7 MO phenotype by co-injecting site-directed mutagenized full-length fzd7 (Fig. 3). These results suggest that fzd7 is required for initial heart development, though we cannot exclude the possibility that it may also be playing a more general role in dorsoventral mesoderm patterning. Fzd7 could be interacting with Wnt11 (Kim et al., 2008; Tao et al., 2005; Witzel et al., 2006), or another wnt ligand such as Wnt3a (Mazzotta et al., 2016), Wnt6 (Gibb et al., 2013; Lavery et al., 2008a, b) or Wnt8c (Ruiz-Villalba et al., 2016; Schneider and Mercola, 2001) during these stages of development.

As suggested, it is possible that the fzd7 morphant cardiac phenotype is a secondary effect of failures in mesoderm specification, patterning, gastrulation, axis formation and tissue separation. We have made efforts to inject embryos at the 4- and 8-cell stages to give as small a convergent extension phenotype as possible to generate normal-looking embryos but with clear heart phenotypes. The results suggest that the effect of fzd7 during early heart development is not secondary to convergent extension defects or mesoderm development, however, this cannot be ruled out completely (Fig. 3).

An interesting feature of the loss-of-function analysis using fzd7 Morpholino and a dominant-negative fzd7 (fzd7 CRD), is that they give different cardiac phenotypes. fzd7 morphants have anterior defects, convergent extension defects and reduction in ntx2-5 expression; whereas fzd7 CRD-capped RNA injections result in embryos with convergent extension defects and cardiac bifida, but no head defects or loss of cardiac markers. Interestingly, it has been shown that the only way to replicate the anterior defect phenotype with a fzd7 CRD construct is to inject the capped RNA into oocytes...
(Medina et al., 2000). This could be because the relevant signalling event has been completed by the time the product of mRNA injected at the 4- or 8-cell stage has been generated. It is possible that if we injected oocytes with fzd7 CRD then we might find embryos showing loss of the heart. Another possibility is that the Morpholino is able to disrupt all Wnt signalling through fzd7 CRD by preventing translation of Fzd7 protein, but fzd7 CRD only disrupts non-canonical signalling in this context. The requirement for coreceptors in canonical signalling may allow the CRD to interact with endogenous fzd7 and any Lrps present allowing the receptor complex aggregates to form. In addition to this, it has been shown to be possible to activate canonical Wnt signalling using CRD constructs (Carron et al., 2003). Perhaps canonical Wnt signalling mediated by fzd7 early on during development is allowed to proceed by the Fzd7 CRD, but then when fzd7 switches to mediate non-canonical signalling, the CRD starts to behave as a dominant-negative. Other possibilities are that the Morpholino may have a broader specificity than thought or that the injected RNA of the fzd7 CRD construct may not be very stable, and thus only provide a short term effect compared to the Morpholino. These possibilities remain to be tested further.

The fzd7 CRD phenotype is very similar to the wnt11-R Morpholino phenotype (Garriock et al., 2005). It has previously been shown that DM-GRASP/alcam expression lies downstream of wnt11-R signalling and that DM-GRASP/alcam can mediate non-canonical wnt signalling effects on morphogenetic movements involved in the developing heart. The DM-GRASP/alcam Morpholino phenotype is also similar to the fzd7 CRD phenotype in that they both lead to a cardia bifida-like phenotype and a thickening of the myocardium. This suggests fzd7 could be mediating the wnt11-R control of DM-GRASP/alcam expression. This needs to be investigated further.
Ruiz-Villalba et al. (2016) suggest a model where periodic switching between proliferation and differentiation within the developing heart is mediated by the periodic and reciprocal activity of the canonical and non-canonical wnt pathways. *fzd7* could be playing a crucial role in this process depending upon the Wnts and other receptors expressed at specific times.

In conclusion, we have shown *fzd7* to be involved in heart development. Further investigation is required to determine the specific wnt(s) it is interacting with at different stages of heart development.

**MATERIALS AND METHODS**

**Embryo manipulation**

All experiments were performed in compliance with the relevant laws and institutional guidelines at the University of East Anglia. The research was approved by the local ethical review committee according to UK Home Office regulations. *Xenopus laevis* embryos were obtained as previously described (Harrison et al., 2004). Staging of the embryos was carried out according to the normal timetable of Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Embryos at the required stages were fixed in MEMFA, washed in PBS, dehydrated in ascending grades of Methanol/PBS, then stored in 100% MeOH at −20°C until processing for single or double in situ hybridisation.

**Constructs**

*fzd7* full-length (*fzd7* FL) and dominant-negative form *fzd7*-cysteine rich domain (*fzd7* CRD) were sub-cloned into pCS2+ at Cla1–Xho1 restriction sites as described in Wheeler et al. (2000). *fzd7* MO titration by RNA in the rescue experiments was avoided by creating a site-directed mutagenesis construct of the full-coding sequence of *fzd7* (*fzd7* SDM) as described in Abu-Elmagd et al. (2006). *fzd3* full-length (*fzd3* FL) and *fzd3* CRD were kind gifts from Peter Klein (University of Pennsylvania). Dishevelled construct (*Dvl1-Delta-N*) was a gift from Roberto Mayor (University College, London) (De Calisto et al., 2005).

**In vitro capped mRNA synthesis and embryo microinjections**

All capped mRNAs of all genes used for RNA injections were prepared according to the manufacturer’s instructions using the SP6 mMessage mMachine Ambion kit (Invitrogen™ AM1340). Anti-sense oligonucleotides, morpholinos (MOs), were obtained and designed by Gene Tools (www.gene-tools.com, Oregon, USA) using the reported sequence for the control morpholino (CMO) (5′-CCTCTTACCTCAATTTATA TA-3′) and *fzd7* MO (5′-GCGGAGTGAGCAGAAATCGGCTGA-3′).

**Fig. 5. Activation of non-canonical wnt signalling rescues *fzd7* CRD-induced cardia bifida.** (A, Ai) Wild-type control embryos showing normal *tnnic* expression in the heart. (B, Bi) *fzd7* full-length (*fzd7* FL) overexpression (500 pg) injected into the dorsal blastomeres (DB) at the 4-cell stage show normal heart expression of *tnnic* despite suffering a severe extension movement defect. (C) Embryos injected with 500 pg *fzd7* CRD show cardia bifida phenotype; note that embryos have normal to mild convergent extension defects. (D) Rescue of the *fzd7* CRD (250 pg) cardia bifida phenotype with 250 pg *fzd7* FL, embryos show normal morphology as well as normal *tnnic* expression. (F) Graph of *fzd7* CRD cardiac bifida phenotype rescue with *fzd7* FL. (E, Ei) Rescue of *fzd7* CRD (500 pg) cardia bifida phenotype with *dishevelled1*-Delta-N (*Dvl1ΔN, 1.25 ng*) indicating that *fzd7* is required for the non-canonical signalling in the heart. (G) Graph of *fzd7* CRD cardiac bifida phenotype rescue with *dvl1ΔN*, panel Gi is the key for the cardia bifida phenotype scoring in G. Magnification 20×.
was linearised with XbaI and transcribed by T7; nks2-5 was linearised with BamHI and transcribed with T7; troponin-IC (tmic) was linearised with Xhol and transcribed with T3; gata6 was linearised with XbaI and transcribed with T7. Promega probe synthesis manufacturing instructions were followed with fzd7 probe labelled with Fluorescence-substituted nucleotide (FI-UTP) and for other heart makers labelled with DIG-substituted nucleotide. Each RNA probe was added to 10 ml hybridisation buffer and stored at −20°C for in situ hybridisation. Single (Harland, 1991) or double (Knecht et al., 1995) in situ hybridisation was carried out as previously described (Abu-Elmagd et al., 2006). Anti-Fluorescein was detected using Fast Red tablets (Kelloff et al., 2006) while carried out as previously described (Abu-Elmagd et al., 2006). Anti-Bryja, V., Andersson, E. R., Schambony, A., Esner, M., Bryjova, L., Biris, K. K., Afouda, B. A., Martin, J., Liu, F., Ciau-Uitz, A., Patient, R. and Hoppler, S. (2008). Frizzled7

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