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***Dkk1* and *Dkk2* Regulate Epicardial Specification during Mouse Heart Development**

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Abstract

Background—*Dkk1* and *Dkk2* interact with LRP5 and LRP6 to modulate canonical Wnt signaling during development, and are known to be expressed in the developing heart. However, a loss-of-function mutation in either gene by itself produces no discernable heart phenotype.

Methods—Using standard husbandry techniques, *Dkk1* null and *Dkk2* null mouse lines were crossed to create double null embryos, which we examined using histological and immunohistochemical methods.

Results—Double null embryos die perinatally, with a gross head phenotype reminiscent of *Dkk1* null embryos. Upon examination of late stage hearts, we observe myocardial defects including ventricular septal defects. At earlier stages, double mutant hearts show myocardial and epicardial hyperplasia. Myocardial hypertrophy is associated with a moderate increase in cell proliferation, but epicardial hypercellularity is not. Rather, the field of proepicardial precursor cells near the liver shows a broadening of expression for the cardiac-specific gap junction protein Connexin 43.

Conclusions—*Dkk1* and *Dkk2* both inhibit Wnt signaling to regulate early myocardial proliferation and each can compensate for the loss of the other in that role. Wnt signaling regulates myocardial proliferation in both heart fields at early stages. Additionally, Wnt signaling is sufficient to increase proepicardial specification as measured by Connexin 43 expression, resulting in a hypercellular epicardium and perhaps contributing to later defects.

Introduction

Congenital heart defects are the most common type of human birth defect, affecting as many as 75 in 1000 live births [1]. Heart defects have been extensively classified and studied, but the molecular basis of heart development is just beginning to be understood, and consequently the causes of many heart defects and diseases have not been defined.

Heart development begins with the specification of cardiogenic cells which take up residence in the lateral plate mesoderm (detailed in [2]). These “first heart field” cells later form primitive heart tubes, comprising an inner endothelial layer and an outer myocardial layer. During subsequent development, a second group of cardiogenic cells migrating from

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the paraxial mesoderm contribute extensively to the heart and are referred to as the second heart field. The third and outermost layer of the heart is the epicardium. The epicardium arises from the proepicardium, an organ derived from splanchnic mesoderm. Beginning at E(mbyronic Day)9.0, proepicardial cells migrate to the surface of the heart, where they spread over the surface, covering it with a single layer of mature epicardium.

Components of many molecular signaling pathways have been found to operate during heart development, including Wnt, Nodal, BMP, TGF β , FGF, Notch, and Hedgehog (reviewed in [3;4]). In particular, great progress has been made in the last decade in exploring the role of Wnt signaling during heart development (for recent reviews, see [5;6]). During heart induction Wnt signaling antagonizes cardiac induction in chicks [7]. During subsequent development, Wnt signals control differentiation and proliferation in cardiomyocytes and other tissues [8;9;10].

Canonical Wnt signaling acts by stabilizing the level of the transcription factor β -Catenin. Therefore removing β -Cat(enin) effectively abolishes these signals. For example, mice null for β -Cat do not form mesoderm and therefore never form hearts [11]. When β -Cat is conditionally removed from the second heart field during development, the right ventricle never forms (but is correctly specified). However, ectopically expressing a constitutively stabilized form of β -Cat under the same conditions results in a hypertrophic ventricle, associated with a modest increase in cell proliferation [9]. In cell culture, a similar experiment resulted in a large accumulation of undifferentiated cardiac progenitors [12]. However, the role of canonical Wnt signaling after induction in the first heart field remains unclear.

Additionally, little is known about the role of Wnt signaling in the development of epicardium. Conditionally abolishing β -Cat using an epicardium-specific Cre resulted in no early developmental cardiac defects [13]. However, later in development several problems related to epicardial proliferation and differentiation became visible.

Wnt signals can be modified by many positive and negative inputs. Dkk1 and Dkk2 are secreted proteins that can act as inhibitors of the canonical Wnt pathway by interacting with Wnt co-receptors LRP5 and LRP6 (reviewed in [14]). Ectopic expression of Dkk1 is sufficient to create cardiogenic potential in chick embryonic mesoderm [7]. Dkk1 and Dkk2 are known to be expressed in the developing heart in partly overlapping patterns [15]. Surprisingly, mice null for either *Dkk1* or *Dkk2* have no apparent cardiac phenotype.

Here we create *Dkk1* and *Dkk2* doubly null mutants to examine further the roles of Wnt signaling in heart development. In this system, canonical Wnt signaling is enhanced in all tissues in response to the lack of Dkk inhibition. We report defects in myocardial and trabecular thickness that are visible at early stages but grow more severe late in development. We also find a multilayered epicardium at early stages that is not associated with increased cell proliferation. Our investigation shows that Wnt signals play a positive role in specifying epicardial precursor cells.

Materials and Methods

Mice

All animal research was performed according to NIH and Public Health Service (PHS) policy and was approved by the *Eunice Kennedy Shriver* NICHD Animal Care and Use Committee. The *Dkk1* and *Dkk2* single null mutant alleles have been described previously [16;17] and were combined using standard husbandry techniques. The presence of both null homozygous alleles was detected using PCR using the following primers (all primers shown

in the 5'-3' orientation): *Dkk1* wt allele (GGG AGC CTG AGT ATA AAG GC, AAG AGT CTG GTA CTT GTT CC); *Dkk1* null allele (GCT CTA ATG CTC TAG TGC TCT AGT GAC, GTA GAA TTG ACC TGC AGG GGC CCT CGA); *Dkk2* wt allele (ACC AAC ATA GCC ACC TAT CTT ACC ATT, CTA GTA GAA CTG GTG GCT TGA CAG AAG); and *Dkk2* null allele (GGT CTC CTG GGT GAC CAA ACC TCT CCT AA, GTA GAA TTG ACC TGC AGG GGC CCT CGA).

Histology

Wholemount embryos were dissected and fixed in 4% Paraformaldehyde (PFA) in PBS overnight at 4°C with shaking. Hearts were dissected in PBS and fixed from 1-4 hours at 4°C using 4% Paraformaldehyde (PFA) in PBS. Samples were then embedded in paraffin blocks, and sectioned at 6 or 8 µm. For histological analysis, sections were stained with hematoxylin and eosin (H&E) (Sigma) using a standard protocol.

Immunohistochemistry

Brightfield—Sections were deparaffinized, rehydrated through an ethanol series and washed in 1 × PBS. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 30 minutes at room temperature. Heat Induced Epitope Retrieval (HIER) was performed using Buffer A (Electron Microscopy Sciences) for 20 minutes utilizing a pressurized de-cloaking chamber (Prestige Medical). The sections were washed and incubated in blocking serum (3%; Vector Laboratories) at room temperature for 60 minutes to block non-specific binding. Thereafter the sections were exposed to primary antibodies: rabbit anti-phospho-Histone H3 (Ser10, Upstate 06-570), anti-pan-Cytokeratin (Santa Cruz sc-8018), anti-DIG AP (Fab fragments, Roche 11 093 274 910), anti-Gata4 (Santa Cruz, sc-25310), and anti-c-Kit (C-19, Santa Cruz sc-168), overnight at 4°C. After primary antibody incubation, sections were washed in PBS, incubated with biotinylated secondary antibodies (Vector Laboratories) and processed with a Vectastain ABC peroxidase kit (Vector Laboratories). The sections were developed by using an AEC chromogen (Zymed), counterstained with Hematoxylin, and mounted with Aqua PolyMount (Polysciences).

Fluorescence—Sections were deparaffinized, rehydrated through an ethanol series and washed in 1 × PBS. HIER was performed as described above and sections were incubated in goat or fetal calf blocking serum (3%) (Vector Laboratories) followed by incubation in primary antibodies: rabbit anti- Phospho-Histone H3 (Ser10) or anti-Mf20 (Developmental Studies Hybridoma Bank), overnight at 4°C. Sections were sequentially exposed to appropriate fluorescein-tagged Alexa Fluor secondary antibodies ([1:250], Molecular Probes). The slides were wet-mounted and counterstained utilizing Vectashield with DAPI (Vector Laboratories) for nuclear staining.

TUNEL Staining

Fluorescein conjugated staining was performed using the “*in situ* Cell Death Kit” (Roche diagnostics), according to manufacturer's instructions.

Microscopic examination

The fluorescent images were captured using an Olympus BX60 microscope fitted with a Zeiss Axiocam MRm camera, using Slidebook v.4.1 software (Intelligent Imaging Innovations, Inc., Denver CO). The brightfield images were captured using the same Olympus microscope fitted with an Olympus Q-Color 6 camera, using QImaging software v. 6.0 (Qimaging Corp., Surrey, BC, Canada). All digital images were processed using Photoshop 6 (Adobe Inc., San Jose, CA).

***in situ* hybridization**

Antisense DIG-labeled *Dkk1* and *Dkk2* probes were synthesized from mouse cDNAs using standard methods. The *Dkk1* probe consisted of a full-length cDNA, while the *Dkk2* probe was made from a 574-bp *NcoI* fragment of *Dkk2* cDNA cloned into pBS. The tissue sections were deparaffinized and rehydrated as described previously [16] with the following modifications. Postfixation, the sections were rinsed and hybridized with the probe overnight at 65°C. The probes were detected using anti-DIG AP antibodies (1:2000) overnight at 4°C, and visualized using BM Purple solution (Roche 11 093 274 910 and 11 442 074 001, respectively), overnight at room temperature.

Results

Doubly null *Dkk1*; *Dkk2* mutants have cardiac defects

Embryos singly mutant for either *Dkk1* or *Dkk2* have no visible cardiac developmental phenotype (data not shown). The effects of each single mutation in other parts of the embryo have been described elsewhere [16;17]. Doubly null *Dkk1*, *Dkk2* embryos die at birth and in appearance phenocopy the *Dkk1* null mutant head defect. An ultrasound analysis of double mutant hearts at E(Embryonic day)18.5 reveals no significant functional impairment (data not shown); however sections at the same stage, sectioning plane, and depth display hypertrophic myocardium, ventricular septal defects (Figure 1A-D), and enlarged atria, an early predictor of heart failure (not shown). The double mutant heart appears slightly smaller than the control at every comparable depth of section.

At E15.5, doubly null *Dkk1*; *Dkk2* embryos show a similar range of defects. The mutant heart appears somewhat smaller at every comparable depth of section. At the same time, the myocardium, trabeculae and ventricular septum appear overgrown and thick compared to control hearts at similar sectioning depths, nearly occluding the ventricular chambers (Figure 1E-H). A ventricular septal defect is occasionally observed at this stage (not shown).

At earlier stages, we observe the emergence of the hypertrophic phenotype in doubly null *Dkk1*, *Dkk2* embryonic hearts. At E10.5, the myocardial wall and trabeculae in double mutants are already noticeably thicker and hypercellular compared to controls in similar sections (Figure 2G-L). Additionally, the epicardium is hypercellular and multilayered, compared to the single-cell layer found in control hearts (arrowheads in L). Also at this stage the endocardial cushions appear somewhat larger and hypercellular, though the endothelial/endocardial lining appears normal. At E12.5, doubly null *Dkk1*, *Dkk2* embryonic hearts also show myocardial and epicardial hyperplasia, though to a lesser extent (Fig. 2A-F). Myocardial overgrowth at this stage is most visible in the enlarged and hypercellular trabeculae (compare Fig. 2C, F). There is a continued appearance of hypercellularity and increased size of the endocardial cushions at this stage, but no abnormality is observed in the later stages discussed above.

***Dkk1* and *Dkk2* transcripts are visible through E15.5 in the heart**

We were curious to know whether the hypercellular and overgrown epicardial and myocardial phenotypes described above in doubly null *Dkk1*, *Dkk2* embryos were due to direct or secondary effects of the mutations. We reasoned that the absence of *Dkk1* and *Dkk2* transcripts in normal hearts after E12.5 would indicate a secondary effect resulting from the initial defects, while the presence of transcripts at such later stages could suggest either possibility. Since no good anti-Dkk1 or -Dkk2 antibodies have been described to date, we performed RNA *in situ* hybridizations on wholemount, normal hearts at E12.5 and E15.5.

Dkk1 and *Dkk2* RNA *in situ* hybridizations performed at E10.5 and E12.5 have been well characterized (e.g. [15]), and reveal overlapping patterns of expression in the heart, including the proepicardium at E10.5, and the endocardial cushions at E12.5. However, in contrast to the previously cited report, in our hands control hearts at E12.5 maintain a strong and apparently overlapping expression of *Dkk1* and *Dkk2* transcripts in the atrial and ventricular myocardium. The staining for both transcripts appears much stronger in the left ventricular myocardium and both atria compared to the right ventricle (Fig. 3A-D). In agreement with the previous report, the outflow tract endocardial cushion within the right ventricle is prominently stained for both transcripts (Fig. 3A, C). At E15.5 these patterns are essentially unchanged but the outflow tract stainings have lost prominence (Figure 3E-H). The differences between our observations and previous studies may be partly explained by our choice of RNA probes (see Materials and Methods).

Hypertrophic cells in mutant myocardium have correct fate

As described above, both the epicardium and the myocardium appear thickened in doubly mutant *Dkk1*, *Dkk2* embryonic hearts at E10.5. However, the epicardial hypertrophy appears to lessen at later stages and finally disappears by E15.5, while the myocardium only becomes thicker through all stages examined. We also know that the myocardium contains a variable population of epicardially derived cells, due to delamination and migration starting at E11.5-E12.5. Therefore, we examined the hypertrophic myocardium in E12.5 double null embryos to determine if the phenotype was due to an increased number of cardiomyocytes, or contained undifferentiated epicardial precursors. Staining with antibodies against the myocardial marker MF20 reveals that the cells in the thickened myocardium and trabeculae are indeed cardiomyocytes (Fig. 4A-B). The myocardial hyperplasticity therefore appears to be due to an increase in the number of myocardial cells in the compact myocardium and the trabeculated myocardium (compare mutants and controls in Fig. 2). The morphology of the cells in the mutant compact myocardium appears normal, but the cells of the trabeculae appear rounded compared to the control (compare Fig. 4A-B).

In mutant epicardium, cells express correct markers but often express multipotent markers. To study the defective epicardium in doubly null *Dkk1*, *Dkk2* embryos, we first stained E10.5 mutants with anti-pan-Cytokeratin antibodies (Fig. 4C-D). This result shows that the mutant epicardium, though multilayered, is properly expressing keratin associated with the epithelial to mesenchyme transition appropriate to epicardial cells at this stage.

In addition to staining for a differentiation marker of the epithelial to mesenchyme transition, we also decided to examine the hypertrophic heart cells for the perdurance of pluripotency. Accordingly, we stained E10.5 doubly null *Dkk1*, *Dkk2* embryos with antibodies against c-Kit, a marker of multipotent stem cells. In double mutant hearts, the epicardium contains large and unusual clusters of c-Kit positive cells between rows of c-Kit negative cells. Such clusters are never observed in the control epicardium, though single cells are occasionally seen (Fig. 4E-F, arrowheads). This evidence suggests that multipotent cells are overrepresented in the mutant epicardium.

Altered cell proliferation found in mutant myocardium but not epicardium

Acting on the inference that the hyperplastic heart tissues resulted from an increased presence of c-Kit positive multipotent cells in the epicardium, we examined doubly null *Dkk1*, *Dkk2* embryos at E10.5 for excess proliferation using anti-phospho-Histone H3 antibodies. We also examined double mutants for reduced cell death using TUNEL staining.

A modest, variable increase (ca. 15-40%) in phospho-Histone H3 staining is visible in the double mutant myocardium, depending on the section and specimen under consideration

(Fig. 5A-B). However, no increase in phospho-Histone H3 staining is observed in the mutant epicardium (compare Fig. 5A, B). TUNEL staining is almost completely absent from the heart at this stage, in both control and double mutant embryos (Fig. 5C-D). Taken together, these results suggest that the myocardial hyperplasia is at least partly due to increased proliferation, while the epicardial phenotype (hyperplasia, multilayering, and multipotent cell clusters) is not.

Expression of a marker for epicardial precursor cells is expanded in double mutant embryos

To address the question of a hypercellular epicardium that does not seem to rise from increased proliferation, yet contains multipotent cell clusters, we next examined the epicardial precursor cells. In normal hearts, proepicardial cells are known to express certain markers such as the cardiac-specific gap junction protein Connexin 43 (CX43).

Accordingly, we stained E10.5 doubly null *Dkk1*, *Dkk2* embryos with anti-CX43 antibodies. Compared to control embryos, double mutants show a drastically increased field of CX43 signal in a broad region that may include a portion of the developing liver (Figure 5E-F, asterisks). Therefore, we concluded that the early hypertrophic phenotype in the double mutant epicardium is due to an over-specification of proepicardial cells, rather than increased proliferation of mature epicardium.

Discussion

Wnt signaling is well known to be critical for proper heart development. Defects are associated with several types of heart disease including Arrhythmic Right Ventricle Cardiomyopathy and Degenerative Aortic Valve Disease [18;19].

Other defects in Wnt signaling lead to lethal defects during development. β -Cat null mutants never develop mesoderm, let alone heart [11]. In chicks, overexpression of selected Wnts prevents cardiac development, while ectopic *Dkk1* expression results in ectopic heart induction [7]. Later aspects of cardiac development such as second heart field migration and correct formation of endocardial cushions and coronary arteries depend on adequate Wnt signaling [9;13;20]. Wnt signaling has been repeatedly shown to increase cell proliferation and inhibit differentiation in the heart and elsewhere.

Dkk1 and *Dkk2* inhibition of Wnt signaling increases myocardial proliferation

In contrast to straight loss-of-function Wnt or β -Cat studies, *Dkk* knockouts result in enhanced Wnt signaling. A similar approach has been employed by expressing a constitutively stabilized β -Cat fragment conditionally in the second heart field or in cultured cells [9;12]. In these cases, defects in myocardial and endocardial cell migration of the second heart field, a lack of differentiation, and increased proliferation were observed.

It is interesting that single *Dkk1* or *Dkk2* knockouts do not result in a heart phenotype. There is evidence that *Dkk2* can either inhibit or agonize Wnt signals depending on local conditions [21;22]. It is also believed that Lrp co-receptor binding to either *Dkk1* or *Dkk2* results in selectively favoring certain Wnt signals, leading to different outcomes in otherwise similar cells. However, our results suggest a more straightforward conclusion: that both *Dkk1* and *Dkk2* inhibit Wnt signaling in the myocardium from early stages. This conclusion is also compatible with the observation that the expression patterns of both Dkks overlap in the developing heart at E10.5 [15]. Therefore it is likely that a lack of one *Dkk* is compensated for by the other under these conditions.

Because increased Wnt signaling inhibits heart induction in chick embryos, we expected mouse heart development in the Dkk double mutant to be significantly impaired. However, early development of the heart proceeds normally despite the putative increase in Wnt signaling. We conclude that either the amount of increase in Wnt signaling is insufficient to block induction, or else the chick results do not hold for the mouse.

Otherwise, our results in the myocardium are in broad agreement with expectations. We report that the myocardium from first and second heart field in double null Dkk1 and Dkk2 embryos is thickened and hypercellular from E10.5-E18.5, or all stages observed. However, our results also indicate that myocardial differentiation is normal. The hypertrophy is associated with a small but visible increase in proliferation, leading us to the obvious conclusion that unrestrained Wnt signaling results in excess proliferation, leading to hypertrophy.

Our observations in later heart development appear consistent with this conclusion. The myocardium appears hyperplastic at E15.5 and E18.5 as well, and we observe ventricular septal defects. In wholemount hearts at E12.5 and E15.5, we see a wide field of expression of both *Dkk1* and *Dkk2* transcripts, suggesting they are expressed at later stages and therefore may still affect proliferation.

Most of the work done to dissect Wnt function in heart development has resulted in lethal phenotypes at early stages, so the role of Wnt signaling in late myocardial development is really unexplored. Our observations that the whole myocardium is hyperplastic and that *Dkk1* and *Dkk2* transcripts are expressed in a large part of the heart through E15.5, suggest a continuous role for Wnt signaling in regulating myocardial proliferation. However, the lack of strong Dkk staining in the right ventricle, which is also hyperplastic, is not consistent with this theory.

It is also possible that the later myocardial hypercellularity might arise from the initial epicardial defect, for two reasons. First, the epicardium is known to contribute cells to the myocardium and second, the epicardium is believed to have a role in regulating myocardial proliferation through FGF signaling [23;24]. In future work, this matter could be analyzed using conditional mutations of β -Cat (knockout or knock-in of the stabilized form) in different tissues such as myocardium or epicardium.

The Dkk double mutant affects epicardial specification rather than proliferation

Less is known about the role of Wnt signaling in the epicardium, and few phenotypes have been reported. Conditionally knocking out β -Cat using the proepicardial-specific Wt-1 Cre results in no early developmental phenotype [13]. However, these mutants die by E15.5 with defects in epicardial cell migration and differentiation into both coronary vasculature smooth muscle and myocardium.

In double null *Dkk1* and *Dkk2* mutants, we report an early role in the specification of proepicardial cells. The multilayered epicardium we observe is not the result of increased proliferation of epicardial cells, but rather by the increased specification of proepicardial cells marked by CX43. The results of Zamora and colleagues [13] show no defects during proepicardial development or specification, suggesting that while Wnt signaling is not necessary for epicardial specification, it is sufficient in competent tissue. This role for Wnt signaling is seemingly in opposition to its known negative roles in cardiac induction and differentiation.

Later in development, we do not observe an epicardial phenotype, suggesting that the matter resolves itself using some unknown mechanism. However, as discussed above, epicardial

defects can affect the myocardium and may be visible late in development. However, we do not have the means to trace the fates of such cells. Future studies using conditional mutations and lineage analysis, could help resolve these questions.

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Figure 1.

Dkk1, *Dkk2* double null embryos have cardiac defects at late developmental stages. **A, B** - Control and mutant heart sections at E18.5 reveal multiple defects including hypertrophic myocardium, trabeculae, and ventral septal defect. **C, D** - Magnified areas of interest shown by boxes in (A, B) respectively. In these close-ups the myocardial thickening is more pronounced, while endocardium and epicardium appear normal at this stage. **E, F** - Control and mutant heart sections at E15.5 reveal similar defects including hypertrophic myocardium and trabeculae. These sections are from a similar depth in the heart. **G, H** - Magnified areas of interest shown by boxes in (E, F) respectively. In these close-ups the myocardial thickening is pronounced. The endocardium and epicardium appear normal at this stage. Mutant refers to the double null genotype, while Control refers to either genetically normal (*Dkk1* and *Dkk2*^{+/+}) animals or heterozygous siblings which are indistinguishable from ^{+/+} animals.

Figure 2.

Dkk1, *Dkk2* double null embryos have cardiac defects at middle and early developmental stages. **A-C** - Heart of a control E12.5 embryo at the level of the outflow tract (A) or atrioventricular endocardial cushion (B), and magnified area of myocardium (C) indicated in the boxed area shown in (B). **D-F** - Heart of a *Dkk1*, *Dkk2* double null E12.5 embryo at the level of the outflow tract (D) or atrioventricular endocardial cushion (E), and magnified area of myocardium (F) indicated in the boxed area shown in (E). The ventricular myocardium appears only modestly thicker in the mutant at this stage. However, the trabeculae are noticeably full and hyperplastic compared to the control. The epicardial cells in the mutant appear more closely spaced than to the control, and are occasionally multilayered (arrowheads). Both endocardial cushions also appear hypercellular and larger than the controls. **G-I** - Heart of a control E10.5 embryo at the level of the outflow tract (G) or atrioventricular endocardial cushion (H), and magnified area of myocardium (I) indicated in the boxed area shown in (H). **J-L** - Heart of a *Dkk1*, *Dkk2* double null E10.5 embryo at the level of the outflow tract (J) or atrioventricular endocardial cushion (K), and magnified area

of myocardium (L) indicated in the boxed area shown in (K). The ventricular and trabecular myocardium appear thick and hypercellular in the mutant compared to the control heart. The mutant epicardium is clearly multilayered (compare I, L, arrowheads). Both cushions also appear to be hypercellular and have larger areas than the controls.

Figure 3.

Dkk1 and *Dkk2* transcripts are expressed in an asymmetrical pattern through stage E15.5 in normal hearts. **A-B** - An E12.5 heart stained for *Dkk1* transcripts, ventral (A) or dorsal (B) aspect. In the ventral aspect the right ventricle is on the left. The pattern of staining appears much stronger in the left ventricle but is visible in the right ventricle. Atrial staining is very faint. **C-D** - An E12.5 heart stained for *Dkk2*, ventral (C) or dorsal (D) aspect. The staining pattern appears almost identical to that of *Dkk1*. **E-F** - An E15.5 heart stained for *Dkk1* transcripts, ventral (E) or dorsal (F) aspect. The staining pattern of *Dkk1* is quite similar to that in E12.5 embryos, but the atrial staining appears more pronounced, especially in the right atrium. **G-H** - An E15.5 heart stained for *Dkk2* transcripts, ventral (G) or dorsal (H) aspect. The staining pattern of *Dkk2* is quite similar to that in E12.5 embryos, but the atrial staining appears more pronounced.

Figure 4.

Dkk1, *Dkk2* double null embryonic hearts express correct differentiation markers but contain ectopic multipotent cells. **A-B** - Control (A) and *Dkk1*, *Dkk2* double mutant (B) hearts at E12.5 stained for the myocardial marker MF-20 (red). **C-D** - Control (C) and *Dkk1*, *Dkk2* double mutant (D) hearts at E10.5 stained for the epithelial marker pan-cytokeratin (brown). Note the thickness and multilayering of the epicardium near the bottom of the mutant image (arrowheads). In control and mutant sections a few cells on the edge of the myocardium are also positive for cytokeratin suggesting epicardial origin. **E-F** - Control (E) and *Dkk1*, *Dkk2* double mutant (F) hearts at E10.5 stained for the multipotency marker c-Kit (brown). Note the large cluster of c-Kit-positive cells in the mutant epicardium near the top of (F, arrowhead). In this system the blood cells also stain brown (marked with asterisks) but do not indicate the presence of multipotent stem cells. Blood cells can be identified through position and morphology.

Figure 5.

Dkk1, *Dkk2* double mutant embryos show increased myocardial proliferation and epicardial specification. **A-B** - Control (A) and mutant (B) embryonic hearts stained for phospho-Histone H3 reveal more positive cells in the mutant myocardium, but not epicardium. The epicardium is marked by white arrowheads, while the myocardium consists of the cells inside the epicardial layer. These results were consistent in multiple subjects. Note that blood cells in this system appear to be stained, but faintly. Blood cells can be identified by position and morphology. **C-D** - Control (C) and mutant (D) embryonic hearts stained with the in situ cell death system (TUNEL, green) show very few cells in either subject to be stained. These results were consistent in multiple subjects. **E-F** - Control (E) and mutant (F) embryonic hearts stained for the multipotent marker CX43 (green) show a dramatically increased field of expression in the mutant, apparently extending into the developing liver (region marked by asterisks).