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Twist Factor Regulation of Non-cardiomyocyte Cell Lineages in the Developing Heart

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Abstract

The heart is a complex organ that is composed of numerous cell types, which must integrate their programs for proper specification, differentiation and cardiac morphogenesis. During cardiogenesis members of the Twist-family of basic helix-loop-helix (bHLH) transcription factors play distinct roles within cardiac lineages such as the endocardium and extra-cardiac lineages such as the cardiac neural crest (cNCC) and epicardium. While the study of these cell populations is often eclipsed by that of cardiomyocytes, the contributions of non-cardiomyocytes to development and disease are increasingly being appreciated as both dynamic and essential. This review summarizes what is known regarding Twist-family bHLH function in extra-cardiac cell populations and the endocardium, with a focus on regulatory mechanisms, downstream targets, and expression profiles. Improving our understanding of the molecular pathways that Twist-family bHLH factors mediate in these lineages will be necessary to ascertain how their dysfunction leads to congenital disease and adult pathologies such as myocardial infarctions and cardiac fibroblast induced fibrosis. Indeed, this knowledge will prove to be critical to clinicians seeking to improve current treatments.

Twist Family Transcription Factors and Heart Development

The complex process of cardiac development begins with the specification of the anterior lateral mesoderm, termed the cardiac crescent, which contributes to the first heart field (FHF) myocardium and a portion of the endocardium. (Lyons 1996; Olson and Srivastava 1996; Sucov 1998; Brand 2003; Prall, Menon et al. 2007). These cells first undergo migration to the embryo midline to form a linear heart tube. This heart tube is composed of two cell layers, an inner layer composed of endocardial (endothelial) cells and the outer being cardiomyocytes. The space between the layers is filled with extracellular matrix (ECM) commonly referred to as cardiac jelly (DeLaughter, Saint-Jean et al. 2011). After initial heart tube formation, pharyngeal mesoderm that lies anterior to the cardiac crescent, which is termed the second heart field (SHF), migrates into the heart tube from both the rostral and caudal ends, adding endocardium and myocardium to the now expanding heart (for review see Kelly 2005; Nakajima 2010; Vincent and Buckingham 2010; Zaffran and Kelly. 2012). As a consequence of the SHF cell contribution, the tube loops to the right and balloons to form common atrial and ventricular chambers, adjoined by a single common

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atrioventricular canal (AVC). Further expansion and remodeling (accompanied by atrial, ventricular, and AVC septation) results in a four chambered heart complete with two distinct AVCs - the right AVC being guarded by the tricuspid valve and the left by the mitral valve. During this remodeling process, cells originating from a developmental structure termed the proepicardial organ (PEO) envelop the heart to form the epicardium, while migratory cardiac neural crest cells colonize the outflow tract (OFT) and facilitate its septation (Snider, Olaopa et al. 2007; Gittenberger-de Groot, Winter et al. 2010). Twist family bHLH factors are dynamically expressed within both the FHF and SHF with numerous gain-of-function and loss-of-function studies showing that these factors are crucial for successful completion of cardiogenesis (Riley, Anson-Cartwright et al. 1998; McFadden, Barbosa et al. 2005; Chakraborty, Wirrig et al. 2010; Barnes, Firulli et al. 2011; Tsuchihashi, Maeda et al. 2011; Vincentz, Barnes et al. 2011).

bHLH proteins make up a class of transcription factors which share two common domains. The first is a motif consisting of basic residues that facilitate DNA binding to a canonical consensus sequence CANNTG, which is termed an E-box. The second is the helix-loop-helix domain, which contains two amphipathic α-helices separated by a loop of variable length. The HLH domains facilitate dimerization via juxtaposition of the hydrophobic faces of the α-helices from two different bHLH proteins. Dimer formation allows for correct positioning of the two basic domains that directly bind DNA (for extensive bHLH review see (Massari and Murre 2000)). Of the hundreds of proteins which contain a bHLH motif (Atchley and Fitch 1997) the Twist family is a small group of transcription factors that contain similar amino acid compositions, with particularly high conservation in the bHLH domain (Castanon and Baylies 2002). In mammals, the Twist family of bHLH transcription factors is composed of six members: *Twist1, Twist2, Hand1, Hand2, Paraxis*, and *Scleraxis;* although homologs of these factors can also be found in phylogenetically distant species such as flies, flatworms, and jellyfish (Gitelman 2007; Barnes and Firulli 2009). While Twist proteins show some diversity in their expression profiles, and thus in the developmental processes they regulate, Twist family members have several key characteristics in common. It is has been well established that Twist bHLH proteins functionally operate either as homodimers or heterodimers with other bHLH factors (Castanon, Von Stetina et al. 2001; Castanon and Baylies 2002; Tapanes-Castillo and Baylies 2004; Barnes and Firulli 2009). Twist family members can heterodimerize with non-Twist bHLH proteins, such as the ubiquitously expressed E-proteins. Dimer partner choice has been shown to dictate factor function (Castanon, Von Stetina et al. 2001; Firulli, Redick et al. 2007) and is affected by elements such as gene dosage, which can alter the ratio of Twist family proteins present in the transcription factor pool inside each cell. Phosphoregulation also plays multiple roles in the regulatory process. The phosphorylation of conserved threonine and serine residues within the first α-helix of Twist family bHLH factors has been demonstrated to affect dimerization partner choice and therefore function. Phosphoregulation can also alter intracellular localization of Twist factors thus modifying dimer capacity (Firulli, Howard et al. 2003; Firulli, Krawchuk et al. 2005; Firulli, Redick et al. 2007; Barnes and Firulli 2009). Collectively, these observations establish the need to not only define where and when Twist proteins are expressed, but also understand the potential partners and the post-translational modifications that collectively drive the formation of tissue-specific transcriptional complexes.

Cardiac Neural Crest Cells

Neural crest cells (NCC) arise from ectoderm at the lateral edge of the neural plate, and extend along the dorsal aspect of the neural tube from the mid-diencephalon through the caudal portion of the developing embryo (Creazzo, Godt et al. 1998; Huang and Saint-Jeannet 2004). These ectodermal cells are induced to undergo an epithelial to mesenchymal

transformation (EMT) by a combination of *Wnt*, bone morphogenic protein (*BMP)*, and fibroblast growth factor (Fgf) signaling, resulting in delamination from the neuroepithelium and migration into the caudal pharyngeal arches (Huang and Saint-Jeannet 2004). Many cell types and structures including facial bones, the peripheral nervous system, and endocrine system all receive heavy contributions from NCC and have been extensively reviewed (Gross and Hanken 2008; Creuzet 2009; Heude, Bouhali et al. 2010; Wang, Chan et al. 2011). cNCC are a small vagal subpopulation of NCC and their migration paths feed them directly into the heart (Stoller and Epstein 2005). During heart development, cNCC populations make essential contributions to the heart via migration into the third, fourth, and sixth pharyngeal arches where they will contribute to remodeling of the aortic arch arteries and cardiac outflow tract (Stoller and Epstein 2005). Upon arrival, cNCC differentiate into smooth muscle cells and pericytes, which participate in septation of the embryonic OFT tract into pulmonary and aortic components (Kirby, Gale et al. 1983; Snider, Olaopa et al. 2007; Nelms and Labosky 2010). Subsequently, the majority of neural crest derived cells in the aorticopulmonary septum undergo apoptosis (Poelmann and Gittenberger-de Groot 1999). The remodeling of the aortic arch arteries is a complex programmed process involving the regression of some arterial structures and the persistence of others. Initially, each pharyngeal arch contains a symmetrically branched artery originating from the single vessel of the early OFT. Through the action of cNCC, this symmetrical arterial network is remodeled into the predominantly left sided vascular pattern seen in adults (Snider, Olaopa et al. 2007). This coordinated regression has been associated with Tgfβ2 signaling, as Tgfβ2-/- mice display aberrant apoptosis in both fourth arch arteries, while lacking normal apoptosis in the right dorsal aorta (Molin, DeRuiter et al. 2002).

If a significant portion of the cNCC fail to reach the OFT, as a result of cell death or a defect in migration, a common congenital abnormality known as persistent truncus arteriosus (PTA) results. In PTA, the OFT fails to septate and results in a single large common vessel. The high incidence of PTA in congenital disorders such as Di George Syndrome implicates NCC dysfunction in the pathology of these diseases (Van Mierop and Kutsche 1986). Other NCC-related OFT abnormalities include double outlet right ventricle (DORV) and overriding aorta. These defects result from the incorrect alignment of the aorta with the left ventricle (LV). During proper alignment, the cardiac tube loops to bring the OFT (distal end) into close proximity with the inflow tract (proximal end). The OFT undergoes septation such that the newly formed aorta gets placed between the left and right AVC in a process called wedging, which ultimately results in correct alignment of the aorta over the developing LV and the pulmonary artery over the right ventricle (Allwork and Anderson 1978; Kirby and Waldo 1995). If wedging occurs incorrectly, the aorta is often displaced to the right, resulting in either DORV, where both the pulmonary artery and aorta are connected to the right ventricle, or in overriding aorta, where the aorta partially connects to each ventricle. Experimental evidence suggests that OFT septation must occur properly to avoid looping defects which result in improper wedging (Yelbuz, Waldo et al. 2002). Indeed, the OFT septum in genetic models of cNCC ablation is often either misaligned with the ventricular septum or incompletely developed (Creazzo, Godt et al. 1998).

Although the processes of aortic arch artery and OFT remodeling are still not well understood, genetic loss-of-function models have revealed a number of proteins that are involved in the regulation of cNCC. These include the transcription factor Pax3, which is thought to play a role in NCC progenitor formation (Olaopa, Zhou et al. 2011), the Wnt/Dvl downstream effector Pitx2 (Kioussi, Briata et al. 2002), and bHLH factor Ets1 (Gao, Kim et al. 2010). Loss of these factors results in aortic arch artery and OFT defects such as DORV, PTA, and transposition of the great arteries (TGA). While transcriptional regulation of the genes discussed above clearly affects cNCC movement and function, many other proteins in the surrounding environment have a non-cell autonomous role to play. For instance *Tbx1*,

which is affected by the chromosomal deletion causing Di George Syndrome, is not expressed in NCC, but can be found in tissue of the pharyngeal arches adjacent to NCC (Snider, Olaopa et al. 2007). Despite this fact, haploinsufficient mice display major defects in the development of the aortic arch arteries, indicating that cNCC take important cues relating to migration and morphogenesis from their environment (Garg, Yamagishi et al. 2001; Hutson and Kirby 2003; Snider, Olaopa et al. 2007). Indeed, the forkhead/winged helix transcription factors Foxc1 and Foxc2 are expressed in endothelial tissues surrounding NCCs and have been shown to be essential for proper OFT and arch artery morphogenesis (Hutson and Kirby 2003). These factors most likely act through transcriptional regulation of secreted molecules, such as Endothelin-1, which is produced by endothelial cells neighboring NCCs (Kurihara, Kurihara et al. 1995). Additional factors that mediate cell-cell signaling within NCC populations include the gap junction protein Connexin43 (*Cx43*) (Reaume, de Sousa et al. 1995), the secreted ligand Semaphorin3c (*Sema3C*) (Feiner, Webber et al. 2001), and its receptor Plexin A2 (Brown, Feiner et al. 2001; Hutson and Kirby 2003). Research on these factors has demonstrated the critical nature of cell-cell interactions and communication during cNCC development. While this fact is well appreciated, future studies are still needed to fully map out the signaling pathways involved and thereby determine what role the Twist family bHLH factors play.

Twist1 Function in cNCC

Twist was first identified in *Drosophila*, as a regulator of mesodermal differentiation and myogenesis (Baylies and Bate 1996). *Twist* and its vertebrate homolog *Twist1*, which are essential for gastrulation, have different biological functions depending on dimer partner choice (Castanon and Baylies 2002; Firulli, Krawchuk et al. 2005; Firulli, Redick et al. 2007; Firulli and Conway 2008). Twist1 is a dynamic member of the Twist family of bHLH transcription factors, which is broadly expressed during development and marks the extra-embryonic, somitic, limb, and pharyngeal mesenchyme. (Chen and Behringer 1995; Fuchtbauer 1995; Vincentz, Barnes et al. 2008). Given this expression pattern, it is not surprising that genetic models reveal a role for *Twist1* in regulating specification and differentiation in developing mesenchyme. Systemic deletion of *Twist1* (see Table 1) in mice results in embryonic lethality at E11.5 due to a spectrum of defects that include failure of the neural tube to close, defects in cranial mesenchyme, pharyngeal arches, somites, and limb buds (Chen and Behringer 1995). This phenotype is consistent with a role for *Twist1* in regulating mesenchyme morphology and behavior during NCC migration and development. Furthermore, this correlates well with what is known about *Twist1* function in neoplastic diseases, where a direct connection between elevated *Twist1* expression and EMT has been found (Hoek, Rimm et al. 2004). During EMT, epithelial cells lose polarity and take on an invasive phenotype via loss of cell adhesion due in part to a loss of the calcium dependent transmembrane protein E-cadherin, which is regulated by Twist1 as well as Snail and Zeb1 (Smit and Peeper 2008). In the context of migratory cNCC, current evidence points to N-cadherin as a major mediator of EMT with Snail, Slug, Id2, and Pinch1 expression specifying EMT competence (Duband, Monier et al. 1995; Martinsen and Bronner-Fraser 1998; Snider, Olaopa et al. 2007). It is not currently clear what role, if any, *Twist1* plays in cNCC EMT.

Upon close examination of *Twist1*$^{-/-}$ mice, it was discovered that the OFT cushions contain amorphic cellular nodules (Vincentz, Barnes et al. 2008). These nodules are of NCC origin, as determined by *Wnt1-Cre* lineage mapping, and at E11.5 strongly express the Twist family members *Hand1* and *Hand2* which are expressed within approximately 50% of the NCC mesenchyme within the OFT. In addition, *Twist1* null mice exhibited a delay in colonization of the OFT cushions, as well as aberrant placement of NCC derivatives in the pericardium and endocardium. These data are consistent with research on Saethre Chotzen Syndrome

(SCS), which is caused by TWIST1 mutations (Howard, Paznekas et al. 1997). The data suggests that *Twist1* is essential for guiding cNCC migration and cell adhesion, although aberrant differentiation may also contribute to the phenotype (Vincentz, Barnes et al. 2008). While a role for *Twist1* in cNCC migration has been established, the details regarding its function are still being investigated. Additional experimentation will be needed to fully characterize the OFT nodules, determine why *Hand*-expressing cNCC are prone to forming nodules, as well as which transcriptional programs modulated by *Twist1* control cNCC morphological and migratory properties.

Hand Factor Function in cNCC

Hand1 and *Hand2* are restricted to both distinct and partially overlapping domains during cardiogenesis, suggesting that these factors have both redundant and unique functions, depending on the spatiotemporal nature of their expression. In mice, prior to E7.5 *Hand1* is expressed within extra-embryonic tissues such as the yolk sac, chorion, and mesoderm with this expression being maintained throughout embryonic development (Cross, Flannery et al. 1995; Cserjesi, Brown et al. 1995; Hollenberg, Sternglanz et al. 1995). Robust *Hand1* expression can be observed during early embryonic development in the septum transversum. By E8.5 *Hand1* expression can also be found within the developing primary heart tube in the region that will form the left ventricle, and pericardium (Barnes and Firulli 2009; Barnes, Firulli et al. 2011). As the heart loops rightward, cardiac expression of *Hand1* is maintained in the outer curvature of the left ventricle myocardium, as well as in the pericardium and septum transversum (Cserjesi, Brown et al. 1995; Firulli, McFadden et al. 1998; Barnes, Firulli et al. 2010). During mid-gestation, *Hand1* is expressed within the left ventricular myocardium, medial cNCC populating the caudal pharyngeal arches, and approximately 50% of the cNCC mesenchyme within the OFT cushions (Vincentz, Barnes et al. 2011). *Hand1* null mice die in utero at E9.5, due to defects in the yolk sac, placenta, vasculature, and heart (Firulli, McFadden et al. 1998; Riley, Anson-Cartwright et al. 1998; Morikawa and Cserjesi 2004). Cardiac defects include failure of complete heart tube fusion and hypoplastic LV, which are likely secondary to placental, yolk sac and vascular defects. Recently, hypomorphic *Hand1* alleles have allowed for analysis of the *Hand1* deficient phenotype at later stages, generating additional insight into the function of *Hand1* during cardiogenesis and further showing the precise genetic balance required for expression of Twist-family proteins. Mice with reduced levels of *Hand1* expression (40% of endogenous) display ventricular hypotrabeculation with a thin LV myocardium and abnormally low expression of the left ventricular markers *Nppa, Cited1*, and *Chisel* (Firulli, McConville et al. 2010). Taken together with myocardial specific ablation models, these results support a primary role for *Hand1* function within cardiomyocytes regulating looping and expansion of the left ventricle (McFadden, Barbosa et al. 2005; Firulli, McConville et al. 2010). Conditional ablation of *Hand1* within NCC does not reveal any developmental defects and may reflect a true functional redundancy with Hand2 in these cells (Barbosa, Funato et al. 2007). Indeed, NCC specific loss of *Hand1* in *Hand2*$^{+/-}$ mice results in a dysregulation of *Msx2, Pax9*, and *Prrx2* expression, as well as hypoplasia of certain pharyngeal arch derived structures such as the mandible. This again demonstrates the critical nature of gene dosage for the Twist family of bHLH factors (Barbosa, Funato et al. 2007). Given this data, it seems likely that interactions and redundancy with additional transcription factors may be masking an unknown role for *Hand1* in cNCC development.

The expression profile of *Hand2* overlaps extensively with *Hand1* and *Twist1* expression within NCC derivatives. Early embryonic *Hand2* expression occurs within the SHF-derived myocardium, OFT NCC, and endocardium. Recent examination of *Hand2* expression reveals that early cardiac *Hand2* expression is restricted to endocardium and underlying pharyngeal mesoderm (Barnes, Firulli et al. 2011). *Hand2* right ventricular myocardial

expression correlates with ingress of SHF pharyngeal mesoderm into the expanding heart tube. *Hand2* is also expressed within PEO and epicardium. Not surprisingly, *Hand2* null mice die in utero from cardiac defects. These embryos display a range of severe cardiovascular malformations including a single ventricular chamber with left-sided properties, a dilated aortic and pericardial sac, and failure of the aortic arch arteries to form, resulting in death at E10.5 (Srivastava, Thomas et al. 1997).

In contrast to *Hand1, Hand2* is known to regulate a large number of important processes in cNCC development. While targeted deletion of *Hand2* in NCC derived tissues results in embryonic lethality at E12.5, pharmacological rescue with the β-agonist isoproterenol allows for analysis of cardiovascular development to birth, as the primary cause of death is a reduction in catecholamine production within the sympathetic nervous system. In pharmacologically rescued embryos conditional ablation of *Hand2* in NCC derived tissues (by crossing with the *Wnt1-Cre* strain) results in alignment defects of the aortic arch arteries and OFT, resulting in DORV with associated ventricular septal defects (VSD) (Morikawa and Cserjesi 2008; Holler, Hendershot et al. 2010). These phenotypic abnormalities are thought to be the result of defects in cell migration, cell-cell communication/adhesion, and cell cycle regulation. Histological analysis shows that fewer cNCC reach the OFT in mutants, and that more of the migrating cells travel independently, whereas cNCC of the control embryos seem to migrate as a coherent sheet. Interestingly, Hand2 function in zebrafish development is required for proper ECM remodeling and the subsequent migration of lateral plate mesodermal cells. Hand2 is thought to regulate this migratory event by maintaining matrix metalloproteinase (MMP) activity, resulting in the diminishment of laminin deposition and proper gut looping.(Yin, Kikuchi et al. 2010) While it is not currently clear if Hand2 regulates ECM remodeling during NCC migration, a microarray screen of RNA collected from murine E10.5 *Wnt1-Cre Hand2* conditional knockout (CKO) whole hearts revealed a set of over 300 differentially regulated genes in the NCC specific *Hand2* deletion embryos, and included *Mmp14*. (Holler, Hendershot et al. 2010). Other candidates related to cell migration included *Pdgf, Itga9, Itgα4, Adam19* and *Cx40. Cx40* was of particular interest since it is a component of cardiac gap junctions, which are major mediators of cell-cell communication in the developing heart. Interestingly, *Cx40* was strongly expressed in wild-type cNCC, but was dramatically down-regulated in *Hand2* CKO cNCC. To determine if Hand2 directly binds the *Cx40* putative promoter, chromatin immunoprecipitation assays were conducted on a *Hand2* expressing rat cardiomyocyte cell line. This pull-down showed that Hand2 binds the proximal Ebox-containing regions of the *Cx40* promoter *in vitro*. Furthermore, a luciferase transactivation assay demonstrated that when co-transfected, *Hand2* and its dimer partner *E12* can modestly up-regulate *Cx40-luciferase* expression, suggesting that Hand2 regulates chemical and electrical cell-cell communication through transcriptional control of gap junction components.

In addition to cNCC migration related factors, many cell cycle related genes are differentially expressed in *Wnt1-Cre Hand2* CKOs (Holler, Hendershot et al. 2010). The most highly regulated of these genes (up 37 fold) was cyclin B1 interacting protein 1 (*Ccnb1ip1*), which is an ubiquitin ligase that promotes the degradation of cyclin B. While the details of its function during development remain unclear, recent malignancy related research suggests that *Ccnb1ip1* plays a role in coordinating the cell cycle with cell migration and invasion (Singh, Nicolas et al. 2007) – functions required for OFT septation and valve formation. Others included *cdk6*, a serine/threonine kinase that regulates the G0 to G1 transition by phosphorylating retinoblastoma protein (pRb) (Malumbres and Barbacid 2005), *Insm1*, a regulator of NCC derived sympathetic neuron development which also interacts with cyclin D1 in the heart (Liu, Wang et al. 2006; Pellegrino, Parrish et al. 2011), and several histones. Interestingly, a number of genes already associated with various aspects of cardiovascular development were found to be differentially regulated in the

Wnt1-Cre Hand2 CKO mice. One down-regulated candidate that was particularly interesting was *Sox11*, since *Sox11* null embryos develop DORV and associated VSDs as well as other OFT defects (Sock, Rettig et al. 2004). Other differentially regulated genes include the transcription factor *Foxc1*, the Notch downstream bHLH affecter and potential Hand dimer partner Hey1 (Firulli, Hadzic et al. 2000; Kokubo, Miyagawa-Tomita et al. 2005; Firulli, Redick et al. 2007), the metalloproteinases *MMP14* and *Adam19*, which are critical for tissue remodeling, and *NF-ATc2* which induces *NF-ATc1*. NF-ATc1 has key functions within the endocardium where Hand2 is also expressed (Zhou, Cron et al. 2002). Collagen type XI a1 (*Col11a1*), which is expressed in non-cartilaginous heart tissue, has been implicated in valve development (Peacock, Lu et al. 2008), while the latent transforming growth factor beta binding protein 1 (*Ltbp1*), has also been implicated in various aspects of cardiogenesis, including OFT septation, endocardial cushion EMT, and valve remodeling (Todorovic, Finnegan et al. 2011). Significantly, both *Col11a1* and *Ltbp1* are down-regulated in the *Wnt1-Cre Hand2* CKOs. The Sonic Hedgehog (Shh) signaling repressor *Gli3* was also down-regulated. *GLI3* mutations in humans are associated with isolated VSDs (Qiu, Gong et al. 2006). Interestingly, Gli3 is downstream of *Hand2* within the developing limb (Charite, McFadden et al. 2000). During limb morphogenesis, Hand2 restricts *Gli3* expression to anterior mesenchyme, while Gli3 restricts *Hand2* to the posterior limb mesenchyme. This genetic interaction establishes a Shh/FGF signaling feedback loop that is essential for proper limb patterning (te Welscher, Fernandez-Teran et al. 2002). Since *Shh* has been demonstrated to be necessary for normal OFT development (Washington Smoak, Byrd et al. 2005), it is possible that Hand2 plays a similar role within cNCC. Given the number of transcripts regulated and the range of developmental processes they are known to function in, it is clear that *Hand2* plays an important role in cNCC development. In order to more precisely define this role, additional experiments are required to determine which genes are regulated directly by *Hand2*, and which are regulated by as of yet unidentified intermediates.

Other Twist Family Members

While *Twist1* and Hand factors are established regulators of cardiac morphogenesis, less is known regarding *Twist2, Paraxis*, and *Scleraxis. Twist2* shares a high level of identity with *Twist1*, as well as significant spatial overlap in its expression profile. Like *Twist1*, embryonic *Twist2* expression predominantly occurs in mesenchymal cell populations and mesodermally derived cartilage (Li, Cserjesi et al. 1995), and is thought to be involved in EMT (Ansieau, Bastid et al. 2008). However, *Twist2* expression temporally follows *Twist1*, and a significant role in cardiogenesis has not yet been described. Similarly, little is known of *Paraxis* and its functions during development. Embryonic expression is first observed at E7.5 in cells that will give rise to the paraxial mesoderm. *Paraxis* is subsequently expressed in the rostral paraxial mesoderm, where it plays an integral role in somitogenesis (Burgess, Cserjesi et al. 1995), but has not been directly linked to cardiogenesis. The final member of the Twist family, *Scleraxis*, is first expressed in mice at E9.5 in somites and mesenchyme of the body wall and limb buds. As the embryo matures this expression becomes restricted to areas of developing cartilage and connective tissue, including heart valves (Cserjesi, Brown et al. 1995). Indeed, *Scleraxis*$^{-/-}$ mouse embryos have thickened valve structures, increased expression of cartilage-associated genes such as *Sox9, Msx1*, and *Snail*, and a disruption of ECM and collagen fiber organization. These observations have lead researchers to conclude that *Scleraxis* is involved in valve precursor cell differentiation and ECM organization (Levay, Peacock et al. 2008). However, few direct transcriptional targets of Scleraxis have been identified (Liu, Watanabe et al. 1997; Espira, Lamoureux et al. 2009), and the extent of its role in cardiogenesis is not well defined. Additional investigation will be required to extend our current understanding.

Twist Family Members in the Epicardium

The epicardium originates from a cluster of cells at the venous pole of the heart termed the proepicardial organ (PEO). Due to a combination of BMP and FGF signaling, aggregates of cells from within the PEO's mesothelial projections undergo an EMT event and migrate to the surface of the developing ventricles, ultimately covering the entire surface of the heart in a caudal to cranial fashion (Komiyama, Ito et al. 1987; Lie-Venema, van den Akker et al. 2007). In mice, this process is initiated by E9.0 and is complete by approximately E11.0 (Komiyama, Ito et al. 1987). Once the epicardial cells have enveloped the heart, multiple waves of epicardial cells undergo a secondary EMT and migrate into the adjacent myocardium. Similar to cNCC EMT, this event is regulated by a combination of paracrine growth factors and proteins such as Slug, Snail, and E-cadherin, with good evidence for Ets1, Ets2, α4 integrin, and WT1 involvement (Lie-Venema, van den Akker et al. 2007). Growth factors implicated in epicardial cell EMT include FGF, PDGF, TGFβ, and VEGF (Morabito, Dettman et al. 2001). Upon entering the myocardium, epicardial progenitor derived cells (EPDCs) are guided to their final destinations by signaling mechanisms that are not yet well understood, but are known to involve PDGF-B, PDGFRβ, Tbx5, Thymosin β4, and the Ets transcription factors. EPDCs will ultimately take up position in interstitial spaces of the ventricles and atria, contributing to the cardiac fibroblast lineage, smooth muscle of the coronary vasculature, and cardiac cushions (Lie-Venema, van den Akker et al. 2007). In chick there is some evidence for EPDC contribution to the coronary endothelium (Perez-Pomares, Carmona et al. 2002), although this has not been substantiated in mice (for a review of this topic see Gittenberger-de Groot and Poelmann 2012). While controversial, evidence also exists for limited EPDC contribution to the myocardium (Zhou, Ma et al. 2008).

Currently little is known about Twist family member function in the epicardium and EPDCs. As one might expect, *Twist1* is expressed in EPDCs undergoing EMT, but is not expressed within the epicardium (Zhou, von Gise et al. 2010). *Hand1* is not expressed within the PEO or epicardium but is expressed within the septum transversum that is directly caudal to the PEO. Interestingly, experiments using the $Hand1^{eGFPCre}$ mutant allele in conjunction with *R26R* lineage mapping shows that the permanently-marked *Hand1* expressing cells within the septum transversum migrate into the forming PEO and subsequently mark the entire epicardium, as well as secondary EMT epicardial-derived cell types such as the coronary smooth muscle, and cardiac fibroblasts (Barnes, Firulli et al. 2010). No endothelial cell contributions were observed, further confirming differences between avian and mammalian epicardiogenesis. Collectively, these experiments identify Hand1 as one of the earliest transcription factors that defines the epicardial lineage. Given the transitory expression of Hand1 in the early precursors of the PEO, it will be interesting to see what role if any it plays in the early specification and or migration of the epicardium, and these experiments are currently underway. As a tool, the $Hand1^{eGFPCre}$ allele allows for conditional gene recombination within the early epicardial lineage (Figure 2). In contrast to *Hand1, Hand2* is robustly expressed within the PEO and epicardium during cardiovascular development but is not expressed within epicardial precursors that reside in the septum transversum, placing Hand1 temporally upstream of *Hand2* in this tissue. Recently, $Hand1^{eGFPCre}$ was used to conditionally delete *Hand2* from *Hand1* expressing cells within the septum transversum, as well as their derivatives (Barnes, Firulli et al. 2011). *Hand2* CKOs display defects in epicardial EMT, a deficiency of cardiac fibroblasts, and increased epicardial apoptosis which results in an incompetent coronary vasculature that leads to embryonic lethality by E14.5 (Barnes, Firulli et al. 2011). This phenotype is recapitulated by E9.5 deletion of Hand2 using the tamoxifen-inducible $WT1^{ERT2Cre}$ allele, which mediates ablation of *Hand2* from the septum transversum, PEO, and lateral mesoderm (Zhou, Ma et al. 2008; Barnes, Firulli et al. 2011). Gene expression analysis of primary epicardial cell cultures from

Hand1^{eGFPCre} *Hand2* CKOs revealed increased levels of the fibronectin receptor *Itgα4* (also shown to be effected in NCC *Hand2* ablation studies) and a greatly lowered ratio of *PDGFRα* to *PDGFRβ*. Interestingly, platelet derived growth factor receptors have been previously shown to function in cell fate and specification of the epicardium after secondary EMT. Epicardial cells expressing *PDGFRα* derive into cardiac fibroblasts where *PDGFRβ* is associated with differentiation into coronary smooth muscle (Smith, Baek et al. 2011) *Hand2* CKOs show marked reduction in *PDGFRα* expression as well as reduced numbers of cardiac fibroblasts. Hand2 regulation of *PDGFRα* regulation is direct, as luciferase assays demonstrated that *Hand2* was able to transactivate the *PDGFRα* promoter (Barnes, Firulli et al. 2011). These data support a novel role for Hand2 in specification of epicardial cell fates post secondary EMT.

In addition to modified PDGFR signaling and cell fate, Hand2 plays a role in the ECM by modulating fibronectin assembly. Fibronectin is normally assembled into a complex structural network, which anchors cell and ECM adhesion molecules such as integrins (Pae, Dokic et al. 2008). In wild type epicardial explants fibronectin is organized into neat bundles that facilitate these interactions, influencing actin dynamics, EMT, and the cell cycle. Additionally, fibronectin regulates the stability and availability of extracellular matrix related factors, such as Tgfβ proteins and growth factors (Sottile, Hocking et al. 1998; Leiss, Beckmann et al. 2008). Although transcriptional and protein levels of fibronectin are unaltered in *Hand2* epicardial CKO mice, fibronectin organization in mutant explants is abnormally uniform and sheet-like (Barnes, Firulli et al. 2011). Furthermore, the fibronectin cell surface receptor *Itgα4* is significantly upregulated in *Hand2* CKO explant cultures. Itgα4 is thought to play a role in adhesion of the epicardium to the underlying myocardium via interaction with vascular cell adhesion molecule 1 (VCAM-1), which is reciprocally expressed with *Itgα4* during cardiogenesis (Kwee, Baldwin et al. 1995; Yang, Rayburn et al. 1995; Pinco, Liu et al. 2001). This suggests that the combination of fibronectin disarray and transcriptional dysregulation of downstream signaling and adhesion components could be a key mediator of the *Hand2* epicardial CKO phenotype. Studies in zebrafish have complemented these observations. In wild type zebrafish embryos, fibronectin deposition is restricted to the basal surface of myocardial precursors, while in *Hand2* mutants, fibronectin assembly is disorganized and no longer localized to a single surface (Trinh, Yelon et al. 2005). Unfortunately, how *Hand2* regulates fibronectin assembly is not yet known. Recent research in zebrafish demonstrated that fibronectin deposition at the midline is initially required for temporally correct migration of myocardial precursors (Trinh and Stainier 2004), and that Hand2 transcriptionally downregulates *fibronectin* expression, to create an environment conducive to fusion of these precursors at the midline (Garavito-Aguilar, Riley et al. 2010). These studies indicate that Hand2 plays an important non-cell autonomous role in zebrafish cardiac development by transcriptionally modulating fibronectin expression and deposition.

Twist Family Members in the Endocardium

The endocardium and myocardium both derive from the anterior lateral splanchnic mesoderm which forms the cardiac crescent (Smith and Bader 2007). The heart tube, which consists of an inner endocardial layer and an outer myocardial layer overlying the intervening cardiac jelly, will grow and expand due to SHF and cNCC contributions. Paracrine signaling originating from the myocardium induces endocardial cells to undergo EMT and migrate into the AVC and OFT, forming the endocardial cushions. Simultaneously, signals originating from the endocardium cue expansion and proper patterning of the myocardium. These signaling pathways include VEGF, Notch, BMP, Wnt, and Neuregulin (Armstrong 2004). Post-EMT, the ECM and mesenchymal cells of the cushions are remodeled from their unorganized primitive state into the highly ordered

structures that constitute mature heart valves (Combs and Yutzey 2009). Given the complex combination of EMT, ECM secretion, and remodeling that must occur in a precise spatiotemporal manner for correct valve development, it is not surprising that valve related deficiencies are present in a majority of all heart defects (Barnett and Desgrosellier 2003).

While little is known of Twist family function in the epicardium, perhaps less is known regarding the endocardium and Twist factors in the above-mentioned steps of valvulogenesis. The limited information that is currently available primarily pertains to *Twist1*, and the role it plays in the endocardial cushions during valve formation. While not detectable in ventricular endocardium, *Twist1* is robustly expressed in endocardial cushions of the AVC during murine mid-gestation (Fig 2), although this expression is down-regulated as the cushions are remodeled into valves. Indeed, by E17.5 *Twist1* expression is either completely absent, or nearly so (Chakraborty, Wirrig et al. 2010). Loss-of-function and gain-of-function experiments in transduced chick endocardial cushion explants have revealed multiple roles for *Twist1* during cushion formation and subsequent valve development. *Twist1* can induce cell proliferation in these cushion explants, promote cushion migration, and affect differentiation marker genes (Shelton and Yutzey 2008). These proposed functions correlate well with information gathered from persistent and overexpression of *Twist1* in mice. In these studies a *CAG-CAT-Twist1* transgene was induced by the endothelial specific *Tie2-Cre*, resulting in stable and persistent overexpression of *Twist1* in the endocardium, and endocardium derived cushions (Chakraborty, Wirrig et al. 2010). Despite increases in area, length, and thickness of AV and OFT valve leaflets, double transgenic animals were viable. Detailed phenotypic analysis revealed abnormally high levels of proliferation in these valves, possibly due to a significant increase in *Tbx20* expression. Indeed, *Tbx20* has previously been associated with proliferation via induction of *N-myc* (Cai, Zhou et al. 2005). *Twist1* overexpression was shown to affect several ECM related genes, possibly accounting for phenotypic abnormalities in cushion remodeling and migration. Dysregulated genes include *Col2a1, Mmp2*, and *Mmp13*, and *Postn* – all of which are significantly upregulated (Chakraborty, Wirrig et al. 2010). Furthermore, a *Col2a1* regulatory element is bound by *Twist1 in vivo*, and can be transactivated *in vitro*. This binding occurs at a conserved E-box in the *Col2a1* first intron. Similarly, *Twist1* has been demonstrated to be capable of binding an E-box in the *Postn* promoter and *trans*-activating a reporter construct (Oshima, Tanabe et al. 2002). This information correlates well with the increased levels of *Postn* expression observed at E17.5 in Twist1 overexpressing mice. Together, these results support a role for endocardial Twist1 in promoting proliferation of cardiac valve progenitors, and subsequently guiding their organization as the endocardial cushions remodel, through regulation of critical ECM proteins.

Hand1 is not expressed in the endocardium or endocardial cushions, and Hand1-lineage analysis shows no endocardial contributions of any cells that have expressed *Hand1* (Barnes, Firulli et al. 2010; Vincentz, Barnes et al. 2011). However, myocardial specific ablation of *Hand1* surprisingly results in hyperplastic endocardial cushions that mature into abnormally thick AV valves (McFadden, Barbosa et al. 2005). This would suggest that Hand1 is involved in a myocardium derived signaling pathway that regulates endocardial cushion cells. However, *Bmp2, Bmp4, Smad6, Smad7*, and *Tgfβ* expression levels are all normal in *Hand1* myocardial specific CKOs, leaving the details of such a pathway undiscovered (McFadden, Barbosa et al. 2005). Unlike *Hand1* and similar to *Twist1, Hand2* is strongly expressed within the endocardium and mesenchymal cells of the endocardial cushions during midgestation. However, no endocardial *Hand2* functions have currently been defined. In *Nkx2.5-Cre Hand2* CKO embryos, the cardiac specific transgene mediates deletion of *Hand2* in a ventricular subset of cells primarily consisting of cardiomyocytes. Interestingly, death occurs two days later than in $Hand2^{-/-}$ embryos, suggesting that the earlier lethality

seen in *Hand2*$^{-/-}$ embryos is due to loss of *Hand2* function in non-cardiomyocyte cell populations. This idea is reinforced by the fact that approximately one third of embryos featuring cardiomyocyte specific loss of *Hand2* via the *cTnT-Cre* survive until E12.5, as compared to 100% lethality by E10.5 in *Hand2*$^{-/-}$ embryos (Morikawa and Cserjesi 2008). When the expression profile of E9.0 Hand2$^{-/-}$ whole hearts was compared to that of *Nkx2.5-Cre Hand2* CKO hearts, it was reported that *Gata4, Has2*, and *Bmp5* were downregulated in the systemic knockout, but not in the *Nkx2.5-Cre Hand2* CKO. These differentially regulated genes could be *Hand2* targets in non-cardiomyocyte cell populations, which contribute to the two-day difference in embryonic viability. Interestingly, all of these genes have been linked to endocardial cushion formation and remodeling. Conditional ablation of *Gata4* within endothelial cells results in an EMT defect that leads to hypoplastic cushions. EMT can be rescued by expression of a mutant *Gata4* that is deficient for interaction with Fog cofactors, but septation of the common AV canal fails (Rivera-Feliciano, Lee et al. 2006). Significantly, Gata factors directly regulate *Hand2* expression during RV development via two conserved consensus sites in a *Hand2* cardiac enhancer (McFadden, Charite et al. 2000), possibly suggesting a positive feedback mechanism maintains *Gata4* expression in the endocardium. Similarly, *Has2*$^{-/-}$ mice have abnormal endocardial cushion formation, with AV septal defects (Camenisch, Spicer et al. 2000), while endocardial cushions fail to form entirely in *Bmp5*$^{-/-}$;*Bmp7*$^{-/-}$ double mutant mice (Solloway and Robertson 1999). Furthermore, many of the genes identified as *Hand2* targets in cNCC and the epicardium are also expressed in the endocardium and mesenchymal cells of the endocardial cushions. Given this information, it is tempting to speculate that *Hand2* may regulate the expression of these targets during the initiation of EMT, AV canal septation and valvulogenesis. Indeed, the extracardiac *Hand2* targets *Cx40, Ltbp1, col11a1, Adam19, Pdgfra*, and *Hey1* have all been implicated in these processes (Gu, Smith et al. 2003; Zhou, Weskamp et al. 2004; Kokubo, Miyagawa-Tomita et al. 2005; Kokubo, Tomita-Miyagawa et al. 2007; Peacock, Lu et al. 2008; Bleyl, Saijoh et al. 2010; Todorovic, Finnegan et al. 2011). Additional tissue specific loss and gain-of-function experiments will need to be completed to determine if *Hand2* similarly interacts with or indirectly regulates these targets in the endocardium.

Summary

Twist family factors are widely expressed during cardiogenesis, and systemic deletion has convincingly demonstrated their essential nature. However, the heart is composed of several distinct cell populations which must integrate during development. The tissue specific nature of Twist protein interactions, and how these interactions affect signaling cascades to facilitate integration, is only just beginning to be explored. Our lab, in conjunction with others, is currently undertaking some of the tissue specific loss and gain-of-function experiments that are necessary to fully understand this dynamic family of transcription factors. A firm understanding of the lineage specific roles that Twist proteins play during embryonic development will be indispensable when analyzing how transcription factor dysfunction leads to congenital disease.

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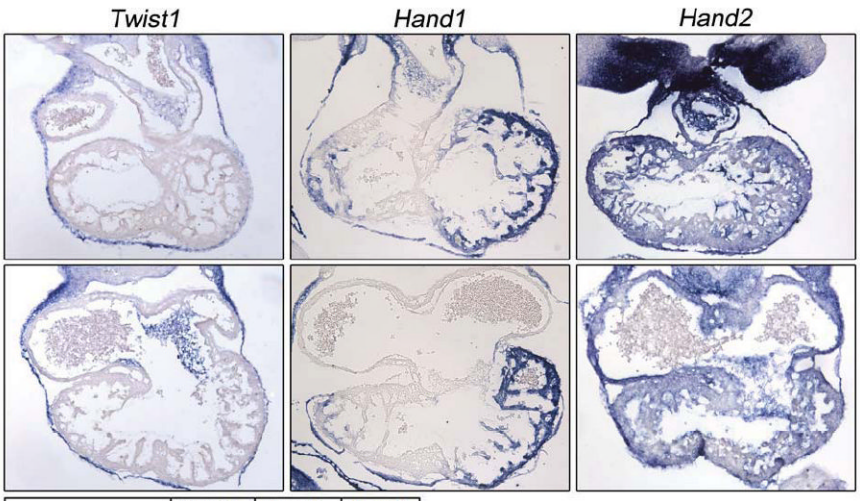

	Twist1	Hand1	Hand2
Myocardium	-	+	+
NCC	+	+	+
Epicardium	-	-	+
Endocardium	-*	-	+

Figure 1. Expression Profile of Twist family members

in situ hybridization of *Twist1, Hand1* and *Hand2* at E10.5 reveal partially overlapping expression. *Twist1* is expressed in the cardiac neural crest, endocardial-derived cushions of the AVC, and the overlying endothelium as well as pericardium. *Hand1* expression is coexpressed with *Twist1* in the cardiac neural crest, the myocardium of the left ventricle, myocardial cuff, and is coexpressed in the pericardium with *Twist1* (and *Hand2*). *Hand2* expression robustly marks the endocardium, cushions of the AVC (coexpressed with *Twist1*), cardiac neural crest (coexpressed with *Twist1* and *Hand1*) and within the epicardium.

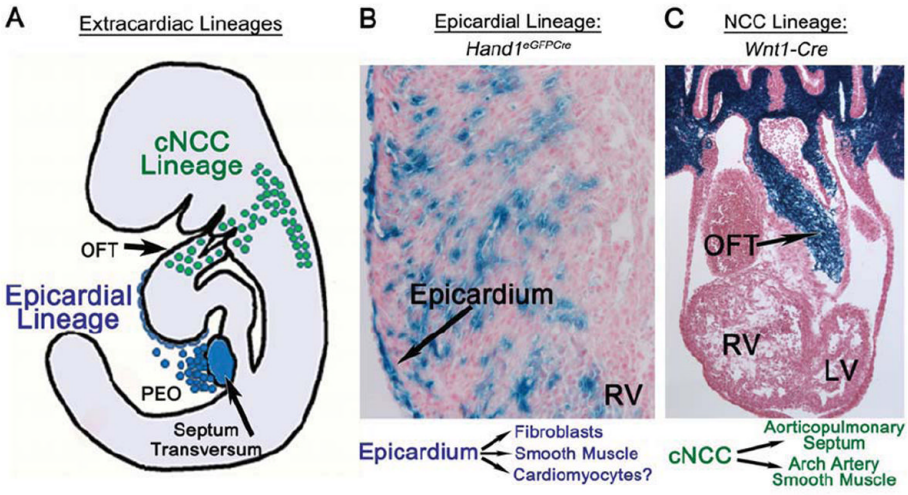

Figure 2. Extracardiac Lineages of the heart

A, diagram of extracardiac lineages. **B**, Epicardial lineage shown by X-gal staining of *Hand1*^{eGFPCre} R26R activation in right ventricle at E15.5 showing epicardial, cardiac fibroblast and coronary smooth muscle expression. **C**, Neural crest cell lineage, shown by X-gal staining of *Wnt1-Cre* R26R activation in OFT at E11.5. PEO, proepicardial organ; OFT, outflow tract; RV, right ventricle; LV, left ventricle; cNCC, cardiac neural crest cell. Left: diagram of epicardial and cNCC lineage contributions to heart development.

Table 1

Cardiovascular defects in select Twist family mouse models

	Cardiovascular Related Expression	Mouse Model	Cardiovascular Related Phenotype	Source
Twist1	NCC, Mesenchyme, Endocardium	*Twist1*$^{-/-}$	Pharyngeal arch defects, aberrant NCC migration	(Chen and Behringer 1995; Vincentz, Barnes et al. 2008)
		Tie2-Cre inducible *Twist1* overexpression (Endothelium)	Abnormal valve remodeling, increased valve thickness	(Chakraborty, Wirrig et al. 2010)
Hand1	NCC, Myocardium, Epicardial Precursors	*Hand1*$^{-/-}$	Abnormal looping, but early embryonic lethality is due to extra-embryonic defects	(Firulli, McFadden et al. 1998; Riley, Anson-Cartwright et al. 1998; Morikawa and Cserjesi 2004)
		Wnt1-Cre; Hand1 CKO (NCC)	No defects (genetic interactions with Hand2)	(Barbosa, Funato et al. 2007)
Hand2	Myocardium, NCC, Epicardium, Endocardium	*Hand2*$^{-/-}$	Single ventricle, failure of aortic arch artery formation, dilation of aortic and pericardial sac	(Srivastava, Thomas et al. 1997)
		Wnt1-Cre; Hand2 CKO (NCC)	Aortic arch artery defects, DORV, VSD	(Morikawa and Cserjesi 2008; Holler, Hendershot et al. 2010)
		Mef2C-Cre; Hand2 CKO (SHF progenitors, Myocardium, Endocardium)	Tricuspid Atresia, PTA, RV hypoplasia	(Tsuchihashi, Maeda et al. 2011)
		Hand1^{Cre}*; Hand2* CKO (NCC, PEO, Epicardium, lateral mesoderm)	OFT septation defects including PTA and DORV, abnormal compaction, and defective epicardialization	(Barnes, Firulli et al. 2011)
		WT1^{ERT2Cre}*; Hand2* CKO (Lateral mesoderm, PEO, Epicardium)	Phenocopies *Hand1*^{Cre}*; Hand2* CKO	(Barnes, Firulli et al. 2011)

NCC, neural crest cells; DORV, double outlet right ventricle; VSD, ventricular septal defect; PTA, persistent truncus arteriosus; OFT, outflow tract; PEO, proepicardium; CKO, conditional knockout