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Heparan sulfate expression in the neural crest is essential for mouse cardiogenesis

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ABSTRACT

Impaired heparan sulfate (HS) synthesis in vertebrate development causes complex malformations due to the functional disruption of multiple HS-binding growth factors and morphogens. Here, we report developmental heart defects in mice bearing a targeted disruption of the HS-generating enzyme GlcNAc N-deacetylase/GlcN N-sulfotransferase 1 (NDST1), including ventricular septal defects (VSD), persistent truncus arteriosus (PTA), double outlet right ventricle (DORV), and retroesophageal right subclavian artery (RERSC). These defects closely resemble cardiac anomalies observed in mice made deficient in the cardiogenic regulator fibroblast growth factor 8 (FGF8). Consistent with this, we show that HS-dependent FGF8/FGF-receptor2C assembly and FGF8-dependent ERK-phosphorylation are strongly reduced in NDST1$^{-/-}$ embryonic cells and tissues. Moreover, WNT1-Cre/LoxP-mediated conditional targeting of NDST function in neural crest cells (NCCs) revealed that their impaired HS-dependent development contributes strongly to the observed cardiac defects. These findings raise the possibility that defects in HS biosynthesis may contribute to congenital heart defects in humans that represent the most common type of birth defect.

1. Introduction

Neural crest cells (NCCs) are ecto-mesenchymal cells that migrate from the dorsal surface of the embryo to populate numerous structures along the dorsoventral axis. Cranial NCCs arise from the forebrain and hindbrain region to populate craniofacial structures as well as pharyngeal arches (PA) 1–3, giving rise to the maxilla, mandible and other structures of the neck and face. The cardiac neural crest (CNC) originates between the otic placode and the 3rd somite in the chick (Kuratani and Kirby, 1992) and populates the third, fourth, and sixth PA as well as the outflow tract (OFT) of the heart. Experimental models that ablate CNCs result in characteristic cardiovascular abnormalities such as failure of OFT septation and aortic arch artery defects (reviewed by (Kirby and Waldo, 1995)). Mouse models with abnormal NCC development also show these characteristic abnormalities (reviewed by Maschhoff and Baldwin, 2000), and several human birth defect syndromes, called 22q11 deletion syndromes, are thought to result from defective NCC development. 22q11 deletion syndromes result in failure of OFT septation (persistent truncus arteriosus (PTA), ventricular septal defects (VSDs), abnormal rotation and alignment of the OFT with the ventricles, double outlet right ventricle (DORV) and tetralogy of Fallot) or abnormal patterning of the aortic arch arteries. Craniofacial findings in these syndromes include cleft palate, abnormal facial features and external ear defects. An important family of factors involved in the formation of these syndromes and required for the survival, migration, proliferation and differentiation of NCCs are the fibroblast growth factors (FGFs) and their receptors (FGFRs). FGF1, FGF2, FGF9, FGF16, FGF20, FGFR1 and FGFR2 are expressed in mouse and chick ventricles during cardiac development (Colvin et al., 1999; Dell'Era et al., 2003; Pennisi et al., 2003; Lavine et al., 2005; Hotta et al., 2008) and activate cardiomyoblast

Abbreviations: VSD, Ventricular septal defect; DORV, Double outlet right ventricle; PTA, Persistent truncus arteriosus; RERSC, Retroesophageal right subclavian artery; NDST, GlcNAc N-deacetylase/GlcN N-sulfotransferase; EXT1, Exostosin 1; NCC, Neural crest cell; FGF, Fibroblast growth factor; FGFR, Fibroblast growth factor receptor; PA, Pharyngeal arch; CNC, Cardiac neural crest; OFT, Outflow tract; HS, Heparan sulfate; HSPG, Heparan sulfate proteoglycan; MAPK, Mitogen-activated protein kinase; ERK, Extracellular signal-regulated kinase; GlcNAc, N-acetyl glucosamine; GlcNS, N-sulfoglucosamine; GlcA, Glucuronic acid; BMP, Bone morphogenetic protein; TGFβ, Transforming growth factor beta.

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proliferation in culture. Mouse mutants made deficient in FGF1 and FGF2 function, however, develop normally and have normal cardiac structure and mass (Zhou et al., 1998; Schultz et al., 1999; Miller et al., 2000). This indicates compensatory activities among FGF family members. FGF8, however, encodes a crucial member of the FGF family, providing survival, mitogenic, anti/-pro differentiation and patterning signals to adjacent tissues as well as autocrine activity (Park et al., 2008). Complete loss of FGF8 function in mice leads to early embryonic lethality (Meyers et al., 1998; Sun et al., 1999; Moon and Capecchi, 2000), and murine FGF8 hypomorphic mutants have a constellation of heart, OFT, great vessel and pharyngeal defects (Abu-Issa et al., 2002; Frank et al., 2002). Mesodermal FGF8 and FGF10 have overlapping roles in heart development (Watanabe et al., 2010). FGF9 is expressed in the midgestation mouse heart and regulates myocardial proliferation and differentiation in vivo (Lavine et al., 2005). FGF9 deficient mice die at birth with an enlarged dilated heart (Colvin et al., 1999).

FGF family members and their receptors require heparan sulfate (HS) for the formation of high affinity FGF- and FGFR-complexes and subsequent signaling (Rapraeger et al., 1991; Yayon et al., 1991). HS is produced by most mammalian cells as part of membrane and extracellular matrix proteoglycans (the HSPGs) (Esko and Lindahl, 2001). The polysaccharide chain grows by exostosin (Ext) copolymerization of GlcAβ1,4 and GlcNAcα1,4 and is modified by one or more of the four NDST isozymes; the N-deacetylase activity of NDSTs removes acetyl groups from GlcNAc residues, which are then converted to GlcNS through the N-sulfotransferase activity. Subsequent modifications of the HS chain by most O-sulfotransferases and a GlcA C5-epimerase depend on the presence of GlcNS residues, making the NDSTs responsible for the generation of sulfated HS ligand binding sites (Lindahl et al., 1998). Mice deficient in EXT1, NDST1, 2-O-sulfotransferase and GlcA C5-epimerase show defective brain morphogenesis, axon guidance defects, craniofacial defects, defective formation of the lacrimal glands, skeletal defects, renal agenesis and eye defects due to simultaneous inhibition of multiple HS-binding factors (Bullock et al., 1998; Inatani et al., 2003; Li et al., 2003; McLaughlin et al., 2003; Grobe et al., 2005; Pan et al., 2006; Pallerla et al., 2007; Pan et al., 2008; Iwao et al., 2009). Mice deficient for the HSPG Glypican3 (GLP3) show defective heart development, as do mice lacking the HSPG Perlecan (Cano-Gauci et al., 1999; Costell et al., 2002; Ng et al., 2009). In humans, mutations in B3GAT3, the gene coding for glucuronosyltransferase-I (GlcAT-I), result in variable combinations of heart malformations, including mitral valve prolapse, VSD, and bicuspid aortic valve (Baasanjav et al., 2011). Importantly, craniofacial defects in NDST1-deficient mouse embryos are consistent with NCC deficiencies and resemble mutants deficient in Sonic hedgehog (SHH) and FGF8 function (Grobe et al., 2005). Therefore, we analyzed these mice for SHH/FGF- and NCC-related cardiac developmental defects, and found that NDST1 null mice indeed show multiple cardiovascular malformations, in large part due to impaired NCC function.

2. Results

2.1. Heart defects in NDST1 deficient embryos

FGF2 signaling and the development of NCC-derived facial and cranial structures are impaired in NDST1 null embryos (Grobe et al., 2005; Pallerla et al., 2007). Therefore, we analyzed E14.5 ($n = 4$) and E18.5 ($n = 7$) NDST1$^{-/-}$ embryos for potential FGF- and NCC-dependent developmental defects of the cardiovascular system. We detected membranous VSD in all E18.5 NDST1$^{-/-}$ mutants (Fig. 1B). Moreover, formation and remodeling of the fourth pharyngeal arch arteries to form the aortic arch and right subclavian artery are extremely sensitive to FGF8 dosage in the pharyngeal ectoderm (Macatee et al., 2003). Consistent with this, we detected retroesophageal right subclavian artery (RERSC) in one E18.5 NDST1 mutant (Fig. 1D), and double outlet right ventricle (DORV) was identified in one out of four E14.5 mutant embryos, indicating that proper alignment and rotation of the OFT were disrupted or delayed (Table 1). These findings provide an explanation for the perinatal lethality of NDST1 null mice, consistent with cyanosis and respiratory distress observed in NDST1$^{-/-}$ neonates (Fan et al., 2000; Ringvall et al., 2000).

2.2. NDST1 expression and HS composition in the mouse embryonic heart

Because all 4 NDST isoenzymes contribute to HS synthesis, and because multiple growth-promoting FGFs bind to HS during cardiogenesis, we first performed semiquantitative RT-PCR to determine FGF-, FGFR- and NDST expression in the E14.5-E18.5 embryonic heart (Table 2). NDST1 was strongly expressed at all stages investigated, whereas NDST3 and NDST4 mRNA expression was generally weaker or absent. The HS-binding fibroblast growth factors FGF1, FGF2, FGF8 and FGF9 as well as their receptors were also widely expressed. However, neither mRNA nor protein expression does strictly predict NDST activity. During biosynthesis, several HS-synthesizing enzymes form a multienzyme complex termed the GAGosome (Esko and Selleck, 2002). GAGosome composition of specific NDST isoforms can vary depending on their relative levels of expression or that of other proteins that may act as chaperones or scaffolding proteins. Therefore, as a functional readout for NDST1 enzyme activity, heart sections were stained with two different HS-specific antibodies, HepSS1 and 10E4. Both antibodies require NDST-generated GlcNS to bind HS. HepSS1 antibodies detected HS localized to various regions of the wildtype embryonic heart, whereas HS expressed in Ndst1 mutant tissues was not detected (Fig. 2). Likewise, we found that HS-staining by 10E4 specifically depended on NDST1 activity, consistent with previous findings (Pan et al., 2006; Pallerla et al., 2007). In E12.5 wildtype embryos, 10E4 stained the developing ventricles and ventricular septae (Fig. 2). 10E4 staining was undetectable in corresponding NDST1 null tissue sections.

Next, HS was isolated from E18.5 wildtype and NDST1 mutant embryos for compositional analysis by quantitative mass spectrometry (Lawrence et al., 2008) (Table 3). Disaccharide analysis revealed strongly increased levels of unsulfated disaccharide D0A0 (68.6% relative abundance in NDST1$^{-/-}$ derived HS versus 51.5% in wildtype derived HS). In contrast, the relative abundance of N-sulfated, 2-O sulfated and 6-O sulfated disaccharides was reduced in the NDST1 null embryo (24.4% versus 40.7%, 13.7% versus 19.1% and 21.2% versus 23%, respectively). These findings confirm that deletion of NDST1 affects O-sulfation in addition to N-sulfation. 10E4 binding to HS requires specific N-sulfated and N-acetylated disaccharide units (van den Born et al., 2005); thus, complete loss of 10E4 binding to NDST1 null tissues despite only moderately reduced N-sulfation confirms specific, NDST1 dependent synthesis of the 10E4 HS epitope (Pallerla et al., 2007).

2.3. NDST1 is a regulator of FGF function and proliferation in the embryonic heart

Cell proliferation depends on FGF function, and the NDST ortholog Sulfateless plays crucial roles in the generation of FGF binding sites in *Drosophila melanogaster* (X. Lin et al., 1999) and in cell culture (Ishihara et al., 1993). FGFs bind and activate alternatively spliced forms of four tyrosine kinase FGF receptors (FGFRs 1–4) (Zhang et al., 2006). FGF activity is regulated by spatial and temporal expression patterns of FGFs and FGFRs, and by the regulated formation of specific ligand-receptor pairs. FGF ligand-receptor pairing is regulated by their binding specificity to HS chains of HSPGs, which in turn depends on specific HS sulfation motifs. These motifs are essential for the formation of signaling-active, trimeric FGF/FGFR/HS complexes. To test for HS-dependent FGF/FGFR assembly that regulates heart development starting at E10.5 (Ostrovsky et al., 2002), heart sections were incubated with FGF and FGFR fused with human IgG Fc domain (FGFR/Fc). Bound FGFR-Fc was then probed with a fluorescent anti-IgG antibody. In E10.5 wild type embryo sections, we observed FGFR1c, FGFR2b, FGFR2c and

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