**Neural crest cell-autonomous roles of fibronectin in cardiovascular development**

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**ABSTRACT**

The chemical and mechanical properties of extracellular matrices (ECMs) modulate diverse aspects of cellular fates; however, how regional heterogeneity in ECM composition regulates developmental programs is not well understood. We discovered that fibronectin 1 (Fn1) is expressed in strikingly non-uniform patterns during mouse development, suggesting that regionalized synthesis of the ECM plays cell-specific regulatory roles during embryogenesis. To test this hypothesis, we ablated Fn1 in the neural crest (NC), a population of multi-potent progenitors expressing high levels of Fn1. We found that Fn1 synthesized by the NC mediated morphogenesis of the aortic arch and differentiation of NC cells into vascular smooth muscle cells (VSMCs) by regulating Notch signaling. We show that NC Fn1 signals in an NC cell-autonomous manner through integrin α5β1 expressed by the NC, leading to activation of Notch and differentiation of VSMCs. Our data demonstrate an essential role of the localized synthesis of Fn1 in cardiovascular development and spatial regulation of Notch signaling.

**KEY WORDS:** Cardiovascular development, Fibronectin, Integrin α5β1, Neural crest, Notch, Lateral induction, Vascular smooth muscle cells

**INTRODUCTION**

Aortic arch arteries (AAAs) route oxygenated blood from the heart to the rest of the body. Malformations in this vascular tree are common causes of morbidity and mortality in patients with congenital heart disease (CHD) (Go et al., 2013; Moon, 2008, 2006). Development of the AAAs occurs similarly in humans and in mice, and results from asymmetrical remodeling of the three pairs of pharyngeal arch arteries (PAAs) that are initially positioned symmetrically around the midline (Conway et al., 2003; Stoller and Epstein, 2005) (Fig. 1A,B). Animal models indicate that defective remodeling of initially well-formed, symmetrical PAAs may cause some forms of CHD, e.g. the Alagille syndrome (High and Epstein, 2008). In this syndrome, as well as in many non-syndromic CHD patients, the AAA abnormalities stem, in part, from defects in derivatives of the 4th pair of PAAs (the left and/or the right). The left 4th PAA gives rise to the aortic arch and the right 4th PAA gives rise to the proximal segment of the right subclavian artery (Stoller and Epstein, 2005). Regression of the left 4th PAA results in the interrupted aortic arch, which is lethal. Regression of the right 4th PAA results in a vascular abnormality called retroesophageal right subclavian artery (RERSA), in which the right subclavian artery originates from the descending aorta on the left, instead of the brachiocephalic trunk on the right (Stoller and Epstein, 2005).

The development of vascular smooth muscle cells (VSMCs) around the three symmetrical pairs of PAAs is necessary to prevent premature regression of these vessels and to facilitate their asymmetrical remodeling (High et al., 2007; Hutson and Kirby, 2007; Yashiro et al., 2007). VSMCs surrounding the PAA endothelium derive from the neural crest (NC) – a population of neuroectodermal stem cells that originate along the anterior–posterior axis at the dorsal margin of the neural tube (Donoghue et al., 2008; Le Douarin and Kalcheim, 1999). NC cells detach from the neural tube and migrate extensive distances along the stereotypical paths characteristic of their axial positions of origin. Upon arrival at their destinations, NC-derived cells participate in the morphogenesis of numerous organs and differentiate into a diverse array of cell types, including neurons and glia of the peripheral nervous system, bones and cartilage of the face, melanocytes of the skin, and VSMCs of the cerebral and pharyngeal vasculature (Crane and Trainor, 2006; Le Douarin and Kalcheim, 1999). NC cells that migrate into the pharyngeal arches 3-6 and into the heart are called cardiac NC cells. The cardiac NC originates from the region of the dorsal neural tube located between the otic pit and the 4th somite (Chan et al., 2004; Hutson and Kirby, 2007).

Studies in chickens and mice show that NC cell fates are influenced by signals emanating from their host tissues (Chen et al., 2012; Donoghue et al., 2008; Ferguson and Graham, 2004; Itasaki et al., 1996; Trainor and Krumlauf, 2000; Trainor et al., 2002a,b). In the pharyngeal arches, Notch signaling from the PAA endothelium mediates differentiation of the adjacent NC cell layers into VSMCs, and the region of active Notch around the PAA endothelium corresponds to the area within which NC cells undergo VSMC differentiation (High et al., 2008, 2007; Manderfield et al., 2012). However, mechanisms that limit the activation of Notch to the few NC cell layers proximal to PAA endothelium are unknown.

In our prior work, we made the unexpected observation that fibronectin 1 (Fn1) mRNA and protein are highly enriched in distinct regions of the mouse embryo, including regions corresponding to the developing NC and the pharyngeal arches 3, 4 and 6 (Mittal et al., 2010). Interestingly, many other ECM glycoproteins are also non-uniformly distributed during embryogenesis (reviewed in Astrf, 2013; Watt and Huck, 2013). This suggests that the highly localized distribution of ECM components during embryonic development generates distinct microenvironments that can influence morphogenetic events in a spatially dependent manner. To test this hypothesis, we conditionally inactivated Fn1 in the NC, and found that NC-synthesized Fn1 regulates AAA morphogenesis and the differentiation of NC cells into VSMCs. Our studies indicated that NC-synthesized Fn1 regulates VSMC differentiation by facilitating Notch signal transduction from the endothelium to the adjacent NC cells. Furthermore, we found that Notch activation...
in the NC cells surrounding the PAA endothelium is limited to the NC cells expressing Fn1.

In order to further understand the mechanisms by which Fn1 regulates activation of Notch and the differentiation of NC cells into VSMCs, we sought to identify receptors on NC cells that could transduce Fn1 signaling. Integrins are a major class of ECM receptors that connect the ECM with the actin cytoskeleton and transduce ECM signals into cells (Assoian and Schwartz, 2001; Giancotti and Tarone, 2003; Hynes, 2002; Schwartz and Assoian, 2001). Integrins are heterodimers comprising α- and β-subunits encoded by different genes; 18 genes encoding α-chains and 8 genes encoding β-chains give rise to 24 distinct integrin heterodimers with distinct and overlapping specificities for ECM ligands (Hynes, 2002). In these strains, Cre expression in the cardiac NC is already evident by the 5th-somite stage, which is ~6 h prior to the onset of Fn1 mRNA expression in that region (Mittal et al., 2010; Stottmann and Klingensmith, 2011). To conditionally ablate Fn1 in the NC and its derivatives, we mated Fnnfloxflox animals harboring either the lacZ (R26R/R26R) or GFP (ROSA26mTmG) reporters in a homozygous state with Fn1+/+; Tfp2aIRESCre/−; P3Pro-Cre+/− mice (Muzumdar et al., 2007; Soriano, 1999). The inclusion of reporters allowed the identification and tracking of NC descendants in control and mutant embryos. The relevant domains of Cre expression in these strains are schematically shown in Fig. S1A-B’. The use of P3Pro-Cre and Tfp2aIRESCre strains resulted in efficient downregulation of Fn1 mRNA (compare Fig. 1C-C′ with 1E-E′) and protein (compare Fig. 1D,D′ with 1F,F′) from the cardiac NC. In these and all other assays presented in this work, NC cells were identified by expression of GFP due to the presence of the ROSA26mTmG reporter allele (Muzumdar et al., 2007). For these and all other experiments involving immunofluorescence, the native GFP and tdTomato fluorescence was extinguished, as described in the Materials and Methods. Fn1 protein remaining around the pharyngeal blood vessels in the mutants (Fig. 1F,F′) is synthesized by the endothelium (arrows in Fig. 1E-E′).

RESULTS

Ablation of Fn1 in the NC results in cardiovascular abnormalities and perinatal lethality

Fn1 mRNA becomes detectable in the cardiac NC at about the 8th-10th somite stage of mouse development, and its expression is maintained in the dorsal neural tube at least until the 23rd somite stage (Mittal et al., 2010). Studies using Fnnfloxflox mice, a strain generated 14 years ago, show that the Fn1 protein is downregulated within 24 h of Cre expression (Sakai et al., 2001).

However, 24 h is a significant fraction of the 19 day gestation period. Thus, to avoid potential problems with the perdurance of Fn1 mRNA or protein after Cre-mediated recombination, we chose mouse lines in which Cre expression in the cardiac NC occurred early enough to ablate Fn1 before Fn1 mRNA was synthesized. Hence, we chose the transgenic P3Pro-Cre line and the knock-in Tfp2aIRESCre strain for our experiments (Li et al., 2000; Macatee et al., 2003). In these strains, Cre expression in the cardiac NC is already evident by the 5th-somite stage, which is ~6 h prior to the onset of Fn1 mRNA expression in that region (Mittal et al., 2010; Stottmann and Klingensmith, 2011). To conditionally ablate Fn1 in the NC and its derivatives, we mated Fnnfloxflox animals harboring either the lacZ (R26R/R26R) or GFP (ROSA26mTmG) reporters in a homozygous state with Fn1+/+; P3Pro-Cre+/− or Fn1+/+; Tfp2aIRESCre+/− mice (Muzumdar et al., 2007; Soriano, 1999). The inclusion of reporters allowed the identification and tracking of NC descendants in control and mutant embryos. The relevant domains of Cre expression in these strains are schematically shown in Fig. S1A-B’.

The use of P3Pro-Cre and Tfp2aIRESCre strains resulted in efficient downregulation of Fn1 mRNA (compare Fig. 1C-C′ with 1E-E′) and protein (compare Fig. 1D,D′ with 1F,F′) from the cardiac NC. In these and all other assays presented in this work, NC cells were identified by expression of GFP due to the presence of the ROSA26mTmG reporter allele (Muzumdar et al., 2007). For these and all other experiments involving immunofluorescence, the native GFP and tdTomato fluorescence was extinguished, as described in the Materials and Methods. Fn1 protein remaining around the pharyngeal blood vessels in the mutants (Fig. 1F,F′) is synthesized by the endothelium (arrows in Fig. 1E-E′).
Cardiovascular phenotypes of the *Fn1*<sup>flox<sup>−</sup>/−; P3Pro-Cre<sup>+</sup> and *Fn1*<sup>flox<sup>−</sup>/−; Tjap2a<sup>IRESCre</sup> mutant embryos were comparable (Fig. 2, Fig. S2A-F and Tables S1 and S2). These mutants rarely survive the neonatal period (552 progeny were genotyped, Table S1) and exhibit a plethora of cardiovascular defects (Table S2). We also used the original *Wnt1-Cre1* strain to complement our experiments (Jiang et al., 2000), but we observed only one incidence of RERSA among 42 *Fn1*<sup>flox<sup>−</sup>/−; *Wnt1-Cre1* embryos. Use of the new *Wnt1-Cre2* strain, which was constructed to avoid the drawbacks of the *Wnt1-Cre1* strain (Lewis et al., 2013), resulted in cardiovascular defects in 4 of 8 *Fn1*<sup>flox<sup>−</sup>/−; *Wnt1-Cre2* mutants (Fig. S2G-L and Table S2). One of these mutants had both RERSA and IAA-B. The difference in the incidence of AAA defects between *Fn1*<sup>flox<sup>−</sup>/−; *Wnt1-Cre1* and *Fn1*<sup>flox<sup>−</sup>/−; *Wnt1-Cre2* mutants is significant, *p*=0.0001 (two-tailed Fisher exact test). The NC is the common domain of Cre expression among the *Tjap2a*<sup>IRESCre</sup>, P3Pro-Cre and *Wnt1-Cre2* strains (Fig. S1A,B,C), indicating that the ablation of *Fn1* in the NC caused the observed phenotypes. Since the *Wnt1-Cre2* strain became available only recently, the majority of data in this paper were generated using the *Tjap2a*<sup>IRESCre</sup> and P3Pro-Cre strains. The phenotypes of mutants generated using these strains were similar; Therefore for clarity, we will designate *Fn1*<sup>flox<sup>−</sup>/−; *Tjap2a*<sup>IRESCre</sup> and *Fn1*<sup>flox<sup>−</sup>/−; P3Pro-Cre embryos as *Fn1*<sup>flox<sup>−</sup>/−; *Cre<sup>+</sup>* mutants and their *Cre<sup>+</sup>* littermate controls, as *Fn1*<sup>flox<sup>−</sup>/−; *Cre<sup>−</sup>* in the main text.

The most common malformations in *Fn1*<sup>flox<sup>−</sup>/−; *Cre<sup>+</sup>* mutants were: (1) aberrant patterning of the aortic arch arteries (compare Fig. 2A,B with D,E; see Fig. S2). The incidence of AAA defects varied from 57% to 82% among the three strains assayed (Table S2); (2) formation of the intracardiac cartilage, similar to that described in Gao et al. (2010) occurred at 89-100% penetrance (Chen et al., 2015); and (3) the membranous ventricular septal defect (VSD; curved arrow in Fig. 2F) 29-42% penetrance (Table S2). Cardiac NC contributes menenchymal cells to the thymus, regulating morphogenesis of this organ (Bockman and Kirby, 1984); consistent with that, we found that 44% of mutant embryos had mild defects in thymus development (Chen et al., 2015). Taken together, these phenotypes are characteristic of many other mouse mutants with defective development or function of the cardiac NC (Hutson and Kirby, 2007). None of these malformations were observed in 267 control embryos examined at E14.5, E16.5 or E18.5.

NC cells arising at the same level of the neural tube as the cardiac NC, also contribute to the cardiac, enteric, sympathetic, parasympathetic and sensory nervous systems (Le Douarin and Kalcheim, 1999). However, with the exception of the palatal shelves, all other NC-derived lineages, such as dorsal root ganglia (n=4/4 mutants), enteric nervous system (n=14/15 mutants) and parasympathetic innervation of the heart and the diaphragm (n=6/6 mutants) developed grossly normally in *Fn1*<sup>flox<sup>−</sup>/−; *Cre<sup>+</sup>* mutants (Fig. S2M-T). Cleft palates were found in all *Fn1*<sup>flox<sup>−</sup>/−; *Tjap2a*<sup>IRESCre</sup> mutants and in 4 out of 5 *Fn1*<sup>flox<sup>−</sup>/−; *Wnt1-Cre2* mutants (Fig. S2U-X). These findings indicate that NC-derived *Fn1* is specifically required for the development of a narrow subset of the cranial NC.

**NC-derived *Fn1* is required for the differentiation of NC cells into vascular smooth muscle cells**

AAA defects in *Fn1*<sup>flox<sup>−</sup>/−; *Cre<sup>+</sup>* mutants generated using the *Tjap2a*<sup>IRESCre</sup>, P3Pro-Cre or *Wnt1-Cre2* strains included RERSA, interrupted aortic arch type B (IAA-B), hypoplastic aortic arch and the right-sided aortic arch (Fig. 2, Fig. S2, Table S2); in 6 of 43 *Fn1*<sup>flox<sup>−</sup>/−; *Cre<sup>+</sup>* mutants, RERSA and IAA-B occurred concurrently. These defects are specific to abnormal development of the 4th pair of PAAs (Stoller and Epstein, 2005) and could be due to either defective PAA formation or defective remodeling of the well-formed PAAs into the AAsas (Conway et al., 2003). Injection of black India ink into the hearts of control and mutant embryos isolated at E10.5 showed that the three pairs of PAAs formed normally (n=3) (Chen et al., 2015). Similarly, examination of histological sections in n=10 mutants and controls 1 day later, at E11.5, indicated that all of the PAAs were well formed in the mutants (e.g. compare Fig. 3A,E). Therefore, the AAA defects observed in our mutants at E14.5-E16.5 were due to pathological regression of the initially well-formed 4th pair of PAAs. These phenotypes are analogous to other mutants with defective development of the cardiac NC (Hutson and Kirby, 2007). In such mutants the originally well-formed 4th pair of PAAs regressed because of either (a) insufficiency in cardiac NC cell population or (b) defective differentiation of the cardiac NC into VSMCs (Conway et al., 2003; Stoller and Epstein, 2005).

Consistent with our previous studies showing that Fn1 was not required for NC migration in global *Fn1*-null mutants (Mittal et al., 2010), the deletion of Fn1 in NC cells did not impair NC migration (compare Fig. S3A-B with C-D′). However, unlike in the global *Fn1*-null embryos, Fn1 synthesized by the NC was not required for NC cell survival (Fig. S3B′,D′), suggesting that other cellular sources of Fn1 regulate NC survival.

At E11.5, population densities of NC-derived cells in the control and mutant pharyngeal arches were comparable (compare Fig. 3A,E; blue; quantified in Table S3). However, the coverage of the 4th pair of PAAs with VSMCs was defective in 17 of 25 *Fn1*<sup>flox<sup>−</sup>/−; *Cre<sup>+</sup>* mutants (compare Fig. 3A-D with E-H; quantified in Fig. S4). Four VSMC markers were assayed, αSMA (*Acta2*), SM22α (*Tagln1*), calponin (*Cml1*) and smooth muscle myosin heavy chain 11 (*Myh11*), and we found coordinated downregulation of all of these markers in cardiac NC-derived cells in *Fn1*<sup>flox<sup>−</sup>/−; *Cre<sup>+</sup>* mutants (Fig. 3E-H). As expected, the development of VSMCs around the dorsal aorta (DA) was not affected in our mutants (Fig. 3A,E), because these VSMCs are derived from the mesodermal progenitors and not from the NC.

Vascular expression of the ECM protein fibulin-1, which is required for the development of the cardiac NC and AAA
morphogenesis (Cooley et al., 2008) was not affected in our mutants (Fig. 3I-I‴, M-M‴) and neither was the expression of laminin \(\gamma-1\) (Fig. 3J-J‴, N-N‴), which is a component of vascular basement membranes (Yurchenco, 2011). We also did not observe differences in activation of SMAD2/3 and SMAD1/5/8 between controls and mutants (Fig. S6). Proliferation and survival of NC cells surrounding the PAAs (Fig. 3K,L,O,P) were also not affected in the mutants, Table S3. Taken together, our data indicate that Fn1 synthesized by NC-derived cells is required for the differentiation of NC cells into VSMCs. Furthermore, these data suggest that the mechanisms of Fn1-mediated NC-to-VSCM differentiation do not involve the regulation of ECM deposition or signaling by the TGF\(\beta\) family of proteins.

Remodeling of the PAAs observed in wild-type embryos involves stage-dependent, stereotypical regression of the right 6th PAA and partial regression of the right 4th PAA, which gives rise to a small segment of the right subclavian artery (Hutson and Kirby, 2007); the left 4th PAA persists and gives rise to the aortic arch. The left 6th PAA persists during fetal development and gives rise to the ductus arteriosus (Hutson and Kirby, 2007). By contrast, in animal models with cardiac NC deficiency, PAA regression occurs at random (Leatherbury et al., 1990; Nishibatake et al., 1987; Olaopa et al., 2011; Porras and Brown, 2008). This leads to variable aberrations in the final configuration of the AAAs, similar to the phenotypes observed in our mutants (Table S2, Fig. 2 and Table S2). Consistent with these findings, we observed that NC differentiation into VSMCs was affected on the left, right or both sides of the mutant embryos (Fig. S4). VSMC deficiency around the left 4th PAA is expected to result in the regression of this PAA, and give rise to the interrupted aortic arch (IAA-B). VSMC deficiency around the right 4th PAA is expected to result in the regression of the right 4th PAA and to give rise to RERSA, and we observed IAA-B, RERSA or both IAA-B and RERSA in \(\text{Fn1}^{\text{flox/−}};\text{Cre}^+\) mutants (Fig. 2, Fig. S2 and Table S2). Taken together, our studies indicate that defective morphogenesis of AAAs in \(\text{Fn1}^{\text{flox/−}};\text{Cre}^+\) embryos result from the reduced differentiation of cardiac NC cells into VSMCs and that the NC-derived source of Fn1 plays a fundamental role in regulating cardiovascular morphogenesis.

NC-synthesized Fn1 regulates the differentiation of NC cells into smooth muscle cells in an NC cell-autonomous manner

On their way to pharyngeal arches, NC cells migrate through the cranial mesoderm that expresses \(\text{Fn1}\) (Mittal et al., 2010; Peters and Hynes, 1996). NC cells directly adjacent to the PAA endothelium within the pharyngeal arches are exposed to endothelial Fn1 (Fig. 1C-D‴, E-F‴). Nevertheless, these paracrine sources of Fn1 are not sufficient to support the differentiation of NC cells into VSMCs and AAA morphogenesis (Fig. 2A,B,D,E, Fig. S2A-K and Fig. 3). Taken together, these data suggest that NC-derived Fn1 mediates differentiation of NC cells into VSMCs in NC cell-autonomous manner. In order to test this hypothesis directly, we isolated NC cells from one of two sources: from a fragment of the neural tube (NT) located between the otic pit and the 4th somite and corresponding with the origin of the cardiac NC (Fig. 4A,B) or from the pharyngeal arches 3-6 containing cardiac NC cells, including those destined to give rise to PAA VSMCs (Fig. 4C,D). In these assays, NC cells were identified by the expression of GFP due
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Fig. 4. Fn1 synthesized by the NC regulates the differentiation of NC cells into smooth muscle cells in vitro. NC cells were cultured under smooth muscle differentiation conditions. (A,B) Cardiac NC cells isolated from control (A) and mutant (B) neural tubes at E8.5. NC cells are GFP+ (green). Note prominent αSMA+ stress fibers (red) in controls (A) and their absence in mutant NC cells (B). Arrows indicate NC cells. GFP+ αSMA+ cells (arrowheads) are mesodermal cells co-isolated with the NC. NC cells from control (C) and from mutant embryos (D) are isolated from the pharyngeal arches 3-6 at E9.5. Arrows indicate NC cells (green), arrowheads indicate mesodermal cells. Calponin (red) is expressed by control but not by mutant NC-derived cells. (E) Analyses of SMC differentiation in NC cells isolated from a single embryo are plotted individually. A total of 813 NC cells were analyzed from 3 Fn1flox−/−;Cre+ mutant embryos and 676 NC cells were analyzed from 3 control embryos. (F) 963 NC cells from 7 Fn1flox−/−;Cre+ control embryos and 892 NC cells from 5 Fn1flox−/−;Cre+ mutant embryos were analyzed. (G) NC cells isolated from the pharyngeal arches at E9.5 were cultured on coverslips coated with the indicated ECM proteins and stained to detect αSMA. Each data point is collected from one cultured embryo. Culture of Fn1-deficient NC cells on plasma or cellular Fn1 did not rescue smooth muscle differentiation in these cells. A total of 973 control and 1175 mutant NC cells plated on collagen 1 were imaged and quantified; 780 control and 847 mutant NC cells plated on pFn1 were imaged and quantified, and 843 control and 1062 mutant NC cells plated on cFn1 were imaged and quantified. Statistics were performed using two-tailed, unpaired Student’s t-test with Welch’s correction for unequal s.d. Means±s.d. are shown. Scale bars: 50 μm.

NC-synthesized Fn1 regulates Notch signaling

Several pathways are known to regulate the differentiation of NC-derived cells into VSMCs around PAAs 3-6 (Arnold et al., 2013; High and Epstein, 2008; Kaartinen et al., 2004; Li et al., 2005; Oh et al., 2005; Vallejo-Illarramendi et al., 2009). Among these, the role of Notch signaling has been particularly well studied (High and Epstein, 2008; High et al., 2008, 2007; Manderfield et al., 2015, 2012). Active Notch is an upstream regulator of NC differentiation into VSMCs (High et al., 2007; Manderfield et al., 2012). Prior experiments indicated that Notch signal is transduced from PAA endothelial cells to the adjacent NC-derived mesenchyme, leading to the expression of Notch target genes Jagged1, Hey1 and Hey2 in the NC. Notch signal is then relayed from one NC cell layer to the next as a result of induction of Jagged1 in these cells – a process termed lateral induction (Edison et al., 2000; Manderfield et al., 2012; Ross and Kadesch, 2004). In this process, Jagged1 expressed by the proximal NC cells binds Notch expressed in next NC cell layer, which activates Notch signaling. The relay of Notch signal through the consecutive NC cell layers leads to smooth muscle gene expression and VSMC differentiation in those NC cells that are marked by the activated Notch (Boucher et al., 2011; Doi et al.,...
However, the Notch signal is not relayed through the indefinite number of NC cell layers, but is confined to a few NC cell layers nearest the PAA endothelium (High et al., 2007). How this relay is terminated is unknown (Manderfield et al., 2012).

We noticed that the expression domains of Fn1 and Jagged1 proteins overlapped and diminished coordinately with increasing distance from the PAA vessel lumen (Fig. 5A-E). The expression of Fn1 mRNA is also enriched in NC layers proximal to the PAA endothelium (Fig. 1C-C″) and this expression pattern corresponds with the mRNA expression of other downstream targets of Notch – Hey1 and Hey2 (Fig. 5J,K and Fig. 6I-J′). These data show that Notch signaling around the PAA endothelium is mainly active within the NC cell layers that express Fn1.

These observations prompted us to ask whether Fn1 regulated Notch signaling in the NC. To answer this question, we investigated the expression of Notch transcriptional targets Jagged1, Hey1 and Hey2, as well as the expression of the active form of Notch – the
Notch intracellular domain (NICD) – in NC cells surrounding the 4th pair of PAAs in Fn1flox−;Cre+ mutants and controls at E11.5. For these analyses, we chose those Fn1flox−;Cre+ mutants in which αSMA expression in the NC around the PAAs was downregulated and used their littermate controls for comparisons (Fig. 5F-Q). Our experiments indicated that the expression of Jagged1 was downregulated in 5 of 6 Fn1flox−;Cre+ mutants with defective VSMC differentiation (compare Fig. 5F,F′ with H,H′). Moreover, compared with the mutants, expression of Jagged1 staining in the mutants was mainly confined to the endothelial–NC cell border (dotted brown line). Endoderm (en) is outlined by the dotted white lines in D,D′ and H,H′. Note that Jagged1 staining in the mutants is mainly confined to the endothelial–NC cell border (dotted brown line). (I,J,M-N) Hey1 and Hey2 mRNAs are downregulated in the mutants (M-N). Compare area between the arrows in J and N. The outlined boxes in J and N are expanded in J′,N′. (K,L,O,P) Expression of Notch1 and Notch3 in NC-derived cells surrounding the PAAs were not affected in the mutants. K,L are serial sections to C-D′. O,P are serial sections to G-H′. Scale bars: 50 μm.

If Fn1 regulates the differentiation of NC cells into VSMCs in a NC cell-autonomous manner, NC cells must express receptors that

Antibodies against the activated form of Notch (NICD) recognize cytoplasmic domains of Notch1 and Notch3. To test whether Fn1

regulated Notch signaling through expression of Notch1 and Notch3, we performed in situ hybridization to detect the expression of these receptors in the mutants with downregulated expression of αSMA and Jagged1. These experiments showed that Notch1 and Notch3 were expressed at similar levels in controls and Fn1flox−;Cre+ mutants, n=3/3, (Fig. 5L,M,P,Q). These findings suggested that Fn1 regulates Notch signaling rather than the expression of Notch receptors.

Taken together, our studies demonstrated that NC-synthesized Fn1 regulates Notch signaling in NC cells around the PAAs, and showed that active Notch signal is confined to the region of NC cells enriched in Fn1 protein. These results are consistent with the idea that the enrichment of Fn1 mRNA and protein in the NC cell layers adjacent to the PAA endothelium imposes a spatial specificity to Notch signaling, and limits Notch signaling and NC-to-VSMC differentiation to this region.

Fn1 regulates activation of Notch and NC differentiation into VSMCs by signaling through integrin α5β1

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transduce Fn1 signals regulating Notch signaling and NC-to-VSMC differentiation. NC cells are known to express a number of Fn1 receptors, including α5-α4- and α5-containing integrin heterodimers (Grazioli et al., 2006; Haack and Hynes, 2001; Testaz et al., 1999). Integron α5β1 is thought to be a major Fn1 signal transducer during early to mid-gestation (George et al., 1997, 1993; Mittal et al., 2013, 2010; Pulina et al., 2014, 2011; Takahashi et al., 2007; Yang et al., 1999, 1993). Therefore, we asked whether integrin α5β1 was required for activation of Notch and differentiation of NC-derived cells into VSMCs. Conditional deletion of integrin α5 in the NC using the same Cre-expressing strains as above resulted in defective differentiation of the cardiac NC cells into VSMCs in 11 of 20 integrin α5 mutants (Itga5flox/Cre−), assayed either by expression of αSMA or calponin (compare red signal in Fig. 6A-C with E-G), quantified in Fig. S5. To test whether Notch signaling in the NC was also dependent on integrin α5, we examined Notch activation in Itga5flox/Cre+ mutants exhibiting defective VSMC differentiation. Similar to Fn1flox/Cre+; Cre− mutants, expression of NICD was limited to the immediate vicinity of the PAA endothelium in 4 of 4 Itga5flox/Cre+; Cre− mutants, whereas in controls, NICD was present in multiple NC cell layers (compare green signal in Fig. 6A,A′ with E,E′). As with Fn1flox/Cre+; Cre− mutants, we found that Jagged1 expression was limited to the immediate vicinity of the PAA endothelium in 5 of 5 integrin Itga5flox/Cre+ mutants (compare Fig. 6D,D′ with H,H′). Consistent with these findings, Hey1 and Hey2 mRNA was downregulated in 4 of 4 Itga5flox/Cre+; Cre− mutants (compare Fig. 6I–J′ with M–N′). Expression of Notch1 and Notch3 was not affected in Itga5flox/Cre+; Cre− mutants with defective expression of αSMA and Jagged1 (Fig. 6K,L,O,P). These findings indicated that expression of integrin α5 by NC cells is required for Notch signaling in the NC and differentiation of NC cells into VSMCs.

To test whether impaired Notch signaling was the cause of defective smooth muscle differentiation in integrin-α5-deficient NC cells, we asked whether activation of Notch in mutant NC cells would restore their ability to differentiate into VSMCs. NC cells isolated from Itga5flox/Cre−; Cre+ mutants lack integrin α5β1 (compare Fig. 7A–A′ with B–B′) and exhibit defective smooth muscle differentiation, assayed by the expression of αSMA or calponin (Fig. 7C,D, quantified in G, ‘not infected’ columns). However, activation of Notch signaling in integrin-α5-deficient NC cells by expression of the Notch1 intracellular domain N1ICD (Tang et al., 2008) – but not of control proteins – restored smooth muscle differentiation in these cells (Fig. 7E–G), indicating that integrin α5 regulates NC differentiation into VSMCs by activating Notch signaling.

Our findings are consistent with the model that NC-synthesized Fn1 regulates the activation of Notch and VSMC differentiation by signaling through integrin α5β1 expressed by the cardiac NC. We hypothesize that as the PAs grow in diameter between E10.5 and E11.5 stages of development, a morphogen secreted by the PAA endothelium induces high levels of Fn1 expression in NC cells adjacent to the PAA endothelium and that the region of Fn1 expression in the NC is determined by the diffusion/threshold levels of this morphogen (Fig. 7H). Alternatively, Fn1 transcription in the NC could be induced by mechanical tension arising due to the increasing hemodynamic shear stress on the endothelium in the growing PAs (Bordeleau et al., 2015; Chiquet et al., 2003; Liu et al., 2000). Regardless of how Fn1 is induced, our studies support the hypothesis that enriched expression of Fn1 in NC cells around the 4th pair of PAs imposes a spatial specificity on Notch activation and VSMC differentiation in the NC, and regulates the complex process of PAA remodeling.

**DISCUSSION**

**Role of Fn1 and integrin α5 in NC development and cardiovascular morphogenesis**

Our previous studies revealed that Fn1 mRNA and protein are expressed in non-uniform, dynamic patterns during embryogenesis (Mittal et al., 2010). The high evolutionary conservation of Fn1 and the essential role of Fn1 in cardiovascular development suggested that enriched expression of Fn1 in specific embryonic regions is biologically significant. The studies described in this paper and in our earlier publication (Chen et al., 2015) reveal requisite, cell type-specific functions of Fn1 in embryogenesis and cardiovascular development.

We used three different strains of mice, the P3Pro-Cre and Wnt1-Cre2 transgenic lines and the Tfap2αMoesC Cre knock-in mice to determine the role of Fn1 synthesized by the NC. These experiments demonstrated that specific expression of Fn1 in the NC is required for the development of the palate and the cardiovascular system. Furthermore, we showed that NC-synthesized Fn1 was specifically required for the asymmetrical remodeling of the PAs into the AAAs by regulating Notch signaling and differentiation of NC-derived cells into VSMCs.

Despite the presence of Fn1 at the interface between the PAA endothelium and NC cells in Fn1flox/Cre−; Cre+ mutants, endothelial Fn1 was not sufficient to promote VSMC differentiation in the adjacent NC, demonstrating the importance of NC cell-specific Fn1. Unlike in global Fn1-null embryos, NC cell survival and proliferation were not affected in the conditional Fn1flox/Cre−; Cre+ mutants, demonstrating that Fn1 synthesized by other embryonic cell types is sufficient to support NC proliferation and survival but not their differentiation into VSMCs.

Studies by Costa-Silva et al. (2009) showed that plating wild-type NC cells on plasma fibronectin-coated surfaces promoted the differentiation of NC cells into smooth muscle cells. Our data support these findings, since we observed only minimal smooth muscle differentiation of wild-type NC cells in the absence of plasma Fn1. However, we show that plasma Fn1 is not sufficient to rescue smooth muscle differentiation in Fn1-null NC cells. Together with the results of NC cell culture, our genetic studies in vivo demonstrate the requirement for the NC cell-specific source of Fn1 in the differentiation of NC cells into VSMCs.

Fn1 is alternatively spliced and we previously showed that the presence of EIII A or EIIIB alternatively spliced exons in Fn1 was necessary for NC development and cardiovascular morphogenesis (Astrof et al., 2007). However, both EIII A and EIIIB forms of Fn1 are present around the PAs (Astrof et al., 2007), and both the PAA endothelium and the NC synthesize EIIIB/EIII A Fn1 (our unpublished data). Thus, differences in the alternative splicing are unlikely to explain why endothelial Fn1 does not compensate for the absence of NC-synthesized Fn1. One would expect that in Fn1flox/Cre−; Cre+ mutants, the total levels of Fn1 are lower at the interface of the PAA endothelium and the NC relative to controls; however, a simple decrease in Fn1 levels cannot explain defects observed in Fn1flox/Cre−; Cre+ mutants, given that a 50% decrease in Fn1 protein levels is sufficient to support normal embryogenesis and adult homeostasis in Fn1+/− mice. Addition of ectopic Fn1 (purified from either plasma or cellular sources) in vitro did not rescue NC-to-VSMC differentiation in Fn1-deficient NC cells, supporting the notion that it is the synthesis of Fn1 by the NC that is important for VSMC differentiation of NC cells, rather than the overall levels of Fn1 protein. These results suggest that cell-autonomous and
paracrine sources of Fn1 could engage different signaling pathways in the NC. Alternatively, differences between Fn1 proteins synthesized by the NC and endothelium could be due to potential variations in cell type-specific post-translational modifications of Fn1.

If NC-synthesized Fn1 signaled in an NC cell-autonomous manner, it would have to engage Fn1 receptors expressed by the NC (Testaz et al., 1999). Previous studies demonstrated that integrin α5β1 is a major Fn1 receptor in early and mid-gestation mouse embryos (Chen et al., 2015; George et al., 1997, 1993; Mittal et al., 2013, 2010; Pulina et al., 2014, 2011; Takahashi et al., 2007; Yang et al., 1999, 1993). Therefore, we used conditional mutagenesis to ablate integrin α5β1 expressed by NC cells in a cell-autonomous manner.

Much of what we know about the roles of Fn1 in cell biology has been gleaned from studies with ectopically supplied purified Fn1 proteins (Bae et al., 2014; Hynes, 1990; Schwartz, 2010). However, recent in vivo studies suggest that in addition to paracrine functions (Yoshino et al., 2014), Fn1 could also signal in a cell-autonomous manner (Cseh et al., 2010; Liu et al., 2010; Serres et al., 2014; Stenzel et al., 2011). By using genetic and cell biological approaches, the studies reported in this paper are the first to demonstrate the requisite, cell-autonomous role of Fn1 in the regulation of cardiovascular development. This discovery bears
significant implications for the fields of regenerative biology and tissue engineering by demonstrating that cell-specific synthesis of ECM can modulate distinct aspects of organ morphogenesis and that simply providing ECM in trans as an adhesive support may not elicit the appropriate cell signaling responses.

**Early and late roles of Fn1-binding integrins in AAA morphogenesis**

In our studies, we used mouse strains, in which the Cre recombinase is expressed early in the NC development (~5 somite stage) (Lewis et al., 2013; Olaopa et al., 2011; Stottmann et al., 2004); this is before the delamination of cardiac NC cells and prior to their arrival into the pharyngeal arches. Thus, our studies specifically address the role of Fn1 and integrin α5 in the differentiation of multi-potent NC cells into VSMCs. By contrast, Turner et al. (2015) used the Sm22α-Cre transgenic line to ablate integrins α5 and αv in smooth muscle lineages, including those derived from the NC. SM22α is a marker of early smooth muscle differentiation. Thus, the downregulation of integrins α5 and αv in Ilga5/Ilga5<sup>SM22-Cre</sup> double mutants has most likely occurred after the onset of VSMC differentiation in the NC, explaining the lack of VSMC differentiation defect in these mutants (Turner et al., 2015). Similarly, Turlo et al. (2012) used the Sm22α-Cre transgenic line to ablate integrin β1 and showed that the expression of β1-containing integrin heterodimers, which include α5β1, in smooth muscle cells was important for maintaining AAA VSMCs and AAA integrity. Taken together with the studies by Turlo et al. (2012) and Turner et al. (2015), our work demonstrates that signaling by Fn1 and integrin α5β1 is essential for both the morphogenesis and maintenance of the AAAs: our studies establish the requisite role of Fn1 and integrin α5β1 in the differentiation of NC cells into VSMCs, thereby regulating the remodeling of symmetrical PAAs into the AAAs. The work of Turlo et al. (2012) and Turner et al. (2015) demonstrated that following the differentiation of NC cells into VSMCs, signaling by α5, αv- and β1-containing integrin heterodimers is essential for maintaining VSMCs and the architecture of the AAA vessel wall.

**The role of Fn1 and integrin α5 in VSMC development depends on the ontogeny of VSMC progenitors**

VSMCs in diverse vascular beds arise from different progenitors. Known embryonic sources of VSMCs include the lateral and paraxial mesoderm, mesothelia and the NC (Jiang et al., 2000; Le Lievre and Le Douarin, 1975; Que et al., 2008; Rinkevich et al., 2012; Wasteson et al., 2008; Wilm et al., 2005). Studies by others and our lab indicated that integrin α5 is not required for the differentiation of mesodermal cells into VSMCs (Liang et al., 2014; Turner et al., 2014, 2015). Furthermore, addition of Fn1 to mesodermal VSMC precursors stimulated their proliferation and the synthetic phenotype and opposed their differentiation into smooth muscle cells (Bae et al., 2014; Hedin et al., 1988; Shi et al., 2014). This contrasts with the roles of Fn1 and integrin α5 in the differentiation of NC cells. Currently, it is not clear why signaling by Fn1 and integrin α5 differs between mesoderm- and NC-derived VSMC progenitors. However, it is widely known that VSMCs from different tissue sources can be distinguished on the basis of their gene expression profiles, the mechanisms regulating their differentiation and in their susceptibility to disease and injury (Cheung et al., 2012; Gittenberger-de Groot et al., 1999; Majesky, 2007; Michel et al., 2012; Pfaltzgraff et al., 2014; Ruddy et al., 2008). Furthermore, differences in genetic regulation of VSMC development between the mesoderm and NC contribute to pathological manifestations of congenital disorders such as Marfan and Loesys–Dietz syndromes, both of which involve aneurisms that develop at the borders of mesoderm- and NC-derived VSMCs (Majesky et al., 2011; Ruddy et al., 2008). In the future, it will be instrumental to determine the mechanisms underlying distinct functions of Fn1 and integrin α5 in the differentiation of VSMCs from mesodermal and NC progenitors.

**Fn1 and integrin α5β1 regulate the differentiation of NC cells into VSMCs by activating Notch signaling**

Activation of Notch signaling in NC cells surrounding the PAAs is essential for the differentiation of NC cells into VSMCs (High et al., 2008, 2007; Manderfield et al., 2012). In this paper, we show that Notch activation is limited to the field of NC cells expressing Fn1 and that Fn1 facilitates activation of Notch by signaling through integrin α5β1. How interactions between Fn1 and integrin α5β1 regulate Notch signaling is not yet clear. However, it is known that signaling by integrins can regulate Notch activation both directly and indirectly. In Drosophila, integrin heterodimers containing β1 regulate Notch activation by regulating its intracellular trafficking or processing (Gomez-Lamarca et al., 2014). Studies in Drosophila also showed that the activation of Notch occurs at the tips of basal filopodia and requires the activation of ezrin, Rac, Scar and actin polymerization (Cohen et al., 2010; De Joussineau et al., 2003). Signaling by Fn1 and α5β1 is well known to activate Rac, the Wave/Scar complex and actin polymerization (reviewed by Huttenlocher and Horwitz, 2011). Thus, ablation of Fn1 or integrin α5 in the NC could interfere with Notch signaling by interfering with actin polymerization, formation of basal filopodia, or by altering the trafficking/processing of Notch. An indirect role of β1-containing integrin heterodimers in the activation of Notch was demonstrated in the chick (Rallis et al., 2010). These studies showed that β1 integrins activate Jagged1 transcription through ILK-mediated stabilization of β-catenin. Stabilized β-catenin directly binds and activates Jagged1 promoters upon translocation into the nucleus (Estrich et al., 2006; Rallis et al., 2010). This is consistent with our observations that Notch signaling is active in cells that express Fn1, and that Notch signaling declines concomitant with the decrease in Fn1 levels.

Taken together, our studies highlight the requisite role of the NC-specific source of Fn1 during morphogenesis of the aortic arch arteries and raise many interesting questions: do NC- and endothelial-derived forms of Fn1 differ from each other? Do paracrine and autocrine sources of Fn1 engage different cellular signaling pathways? What are the mechanisms regulating Fn1 expression in the NC and what are the intracellular pathways by which Fn1 and integrin α5β1 regulate Notch signaling?

**MATERIALS AND METHODS**

**Mouse strains and genotyping**

Details of mouse strains used can be found in supplementary Materials and Methods. Genotyping was performed using PCR primers as described previously (Mittal et al., 2010; Sakai et al., 2001; van der Flier et al., 2010). All embryos up to E10.5 were staged by counting somites, older embryos were staged by the day of vaginal plug. All animal studies were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

**Histology**

Histology, immunohistochemistry and β-gal staining were performed as described previously (Chen et al., 2015; Liang et al., 2014). For more details about the procedures and antibodies, see supplementary Materials and Methods.
In situ hybridization

In situ hybridization was carried out using Fn1 (316951), Hey1 (319021), Hey2 (404651), Notch1 (404641) and Notch3 (425171) RNA probes synthesized by Advanced Cell Diagnostics (ACD). Detection was done using RNAasecope 2.0 FFPE Assay Brown kit according to the manufacturer’s protocol (ACD). After the signal was developed, sections were counterstained with Gill’s hematoxylin.

Quantification of vessel coverage by VSMCs

VSMC coverage (Figs S4 and S5) was determined using coronal sections from control and mutant embryos dissected at E11.5. Quantification of 4-18 sections per pharyngeal arch per embryo was performed using ImageJ software. Equivalent numbers of sections were analyzed for each set composed of one control and one mutant embryo and 5-6 sets of control and mutant embryos were analyzed per experiment. To determine VSMC coverage, we calculated the fraction of vessel perimeter occupied by αSMA+ or SM22α signal.

Neural tube explant culture and immunofluorescent staining of NC cells

Control and mutant embryos were obtained by crossing Fn1+/−; Tfap2aRREScw females with Fn1+/−; ROSA26+/mG male mice. E8.5 embryos (15 somites) were dissected in PBS. To obtain integrin-α5-deficient NC cells and their controls, we crossed Itga5−/−; P3Pro-Cre females with Itga5+/−; ROSA26+/mG male mice. The neural tube (NT) from the otic placode to the fourth somite was dissected out and incubated in collagenase/disperse (100 μg/ml) and then longitudinally plated on 12 mm glass coverslips pre-coated with 100 μg/ml collagen type I (BD Biosciences, 354249) in 4-well plates (Thermo Scientific, 12-566-350). To induce smooth muscle differentiation, explants were cultured for 3 days at 37°C and 5% CO2 in high glucose DMEM (Invitrogen) containing 10% fetal bovine serum (BenchMark FBS, Gemini Bio-Products).

To culture NC cells from the pharyngeal arches 3-6 isolated from E9.5 mouse embryos, embryos were incubated with 150 μg/ml collagenase/ disperse for 3 min. Surface ectoderm was peeled off and pharyngeal arches 3-6 were dissected out, plated as above, then cultured in NC self-renewal medium for 4 days (Bixby et al., 2002). On the 5th day, NC cultures were digested using 0.25% trypsin into single cells and plated on 12 mm glass coverslips pre-coated with either 150 μg/ml collagen I, 10 μg/ml plasma Fn1 (BD Biosciences, 356008) or 10 μg/ml cellular Fn1 (Sigma, F2518). We confirmed that cellular Fn1 but not plasma Fn1 contained the EIIIA domain by western blotting using anti-EIIIA antibody (Sigma, F6140).

Smooth muscle differentiation conditions and quantification

To induce smooth muscle differentiation, cells were cultured in DMEM containing 10% FBS at 37°C and 5% CO2 until all cells attached (2 h). After this, NC cells were either left in the medium for the additional 48 h or treated with adenoviruses (see below) for 2 days. Cells were then fixed in 4% PFA for 20 min at room temperature, washed and blocked for 30 min with 10% normal donkey serum in PBS/0.05% Tween-20. Cells were incubated overnight at 4°C with primary antibodies diluted in blocking buffer. After a thorough washing step the next day, cells were incubated for 1 h at room temperature with secondary antibodies and mounted with ProLong Gold antifade. Images were captured with a Zeiss Axiovert inverted fluorescence microscope.

NC cultures were stained to identify NC-derived cells (GFP+, due to the presence of ROSA26mG reporter allele), smooth muscle cells (αSMA or calponin 1) and DAPI (nuclei). For infected cultures, cells were also stained to identify infected cells using antibodies directed against either the FLAG or V5 tags (for further details see supplementary Materials and Methods). All cells in each well were photographed at 20× magnification and coded to conceal the identity of NC cell genotypes. Following quantification, the identity of the samples was decoded and results were analyzed using Prism 6 software (GraphPad). To quantify differentiation of untreated control and integrin-α5-deficient NC cells or NC cells infected with control or N1ICD-encoding viruses, we performed at least four independent experiments, all plotted in Fig. 7G.

Adenoviral infection

Adenoviruses expressing V5-tagged Notch 1 intracellular domain (N1ICD) were produced by the Viral Vector Core at the Maine Medical Center Research Institute (Tang et al., 2008). The N1ICD virus stock concentration was 1.3×1013 viral particles/ml. Control adenoviruses were produced using RAPAd CMV adenoviral expression system (Cell Biolabs, San Diego, CA, USA). Stock adenoviral concentration was 4×1011 pfu/ml. Viral stock solutions were diluted 1:1000 in DMEM containing 10% FBS and 300 μl was used to infect the cells. Viruses were incubated with NC cells at 37°C and 5% CO2 for 48 h. Cells that were then fixed for 20 min with 4% PFA at room temperature, washed with PBS and stained using antibodies against GFP, αSMA or calponin, and V5 or FLAG tags.

Statistica

Data were analyzed by two-tailed, unpaired Student’s t-tests or by two-way ANOVA, as indicated in figure legends utilizing Prism 6 GraphPad software. A P-value of less than 0.05 was considered significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceived and designed experiments: X.W. and S.A. Performed experiments: X.W. Analyzed data and generated figures: X.W. and S.A. Wrote the paper: X.W. and S.A.

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Supplementary information

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