Neurofibromin modulation of ras activity is required for normal endocardial-mesenchymal transformation in the developing heart

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SUMMARY

Endocardial cushions are the precursors of the cardiac valves and form by a process of epithelial-mesenchymal transformation. Secreted growth factors from myocardium induce endocardial cells to transform into mesenchyme and invade the overlying extracellular matrix. Here, we show that the product of the Nf1 neurofibromatosis gene is required to regulate this event. In the absence of neurofibromin, mouse embryo hearts develop overabundant endocardial cushions due to hyperproliferation and lack of normal apoptosis. Neurofibromin deficiency in explant cultures is reproduced by activation of ras signaling pathways, and the Nf1−/− mutant phenotype is prevented by inhibiting ras in vitro. These results indicate that neurofibromin normally acts to modulate epithelial-mesenchymal transformation and proliferation in the developing heart by down regulating ras activity.

Key words: Neurofibromatosis type I, Cardiac valves, Neural crest, Cardiogenesis, Mouse

INTRODUCTION

Von Recklinghausen neurofibromatosis is a common genetic disorder resulting in abnormal cellular growth control and benign and malignant tumors. Neurofibromas, benign nerve sheath tumors composed mostly of Schwann cells, and hyperpigmented lesions (cafe au lait spots) characterize the syndrome and underscore the association between neurofibromatosis and neural crest derivatives (Gutmann et al., 1997; Gutmann and Collins, 1993). Abnormal cellular growth control is present in both neural-crest- and non-neural-crest-derived structures and patients are at increased risk for neurofibrosarcomas, astrocytomas, optic gliomas, pheochromocytomas, as well as for leukemias and rhabdomyosarcomas. Von Recklinghausen or Type I neurofibromatosis is caused by mutations in the NF1 gene that encodes neurofibromin and NF1 is considered a tumor suppressor gene. Loss of heterozygosity for NF1 has been demonstrated in several malignant tumors and myeloid disorders, and in some neurofibromas (Bollag et al., 1996; Sawada et al., 1996; Serra et al., 1997; Side et al., 1997).

The molecular pathways regulated by neurofibromin are only partially understood. It is a large protein (2818 amino acids) which includes a GTPase-activating protein (GAP) related domain (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990). Neurofibromin can act to enhance the intrinsic GTPase activity of ras bound to GTP, thus downregulating activated ras signaling. In Drosophila, NF1 appears to regulate a cyclic-AMP-dependent protein kinase A pathway and to affect a cellular potassium current in a ras-raf independent fashion (Guo et al., 1997; The et al., 1997). Hence, modulation of either of these pathways (or others) may account for abnormal growth regulation in affected tissues.

Strains of mice have been developed in which the Nf1 gene has been inactivated in order to provide animal models for the study of neurofibromin deficiency in mammals (Brannan et al., 1994; Jacks et al., 1994). Heterozygous animals are at increased risk for the development of secondary tumors, consistent with the model of NF1 as a tumor suppressor gene. Homozygous deficient embryos die in mid gestation (approx. E14) and exhibit signs of cardiac dysfunction including peripheral edema and venous hemorrhages. Histologic analysis revealed an overabundance of tissue in the region of the cardiac outflow tract, a thinned myocardium and abnormal cardiac morphogenesis including a failure of proper conotruncal rotation resulting in double outlet right ventricle. This morphogenic defect has been associated with human syndromes and animal models of abnormal cardiac neural crest function (reviewed by Epstein, 1996). Cardiac neural crest cells are known to migrate to and populate the outflow tract of the developing heart and are required for proper septation of the single great vessel emerging from the embryonic heart, the truncus arteriosus, into the aorta and pulmonary artery (Kirby et al., 1983). The Nf1 knockout cardiac defect was therefore proposed to be related to neural crest dysfunction (Brannan et al., 1994; Jacks et al., 1994).

At E9-9.5 of gestation, the mouse heart consists of two epithelial layers, the endocardium and the myocardium that are separated by an abundant acellular matrix (the cardiac jelly). Between E9.5-E10.0, endocardial cells in the atrioventricular
and outflow tract regions become mesenchymal and invade the cardiac jelly forming the endocardial cushions through a process of epithelial-mesenchymal transformation. The formation of the endocardial cushions is restricted to the atrioventricular (AV) canal and ventricular outflow tract (OT) regions of the primitive heart. This tissue represents the primordia of the valve leaflets and membranous septa of the future four-chambered heart (Lamers et al., 1995). The transformation of the cardiac endothelium into mesenchyme depends upon a developmental signal from the associated myocardium that includes TGFβ (Potts and Runyan, 1989; Potts et al., 1991). Using explanted chick endocardial cushions cultured on collagen gels, Runyan et al. (1990) showed that epithelial-mesenchymal transformation is inhibited by staurosporine and genistein, inhibitors of protein kinase C and tyrosine kinases, respectively. The transformation process is associated with increased intracellular [Ca2+] and is sensitive to pertussis toxin, a G-protein inhibitor.

In this report, we demonstrate that the overabundant tissue that accumulates and obstructs blood flow in the hearts of Nf1-deficient mouse embryos results from abnormal epithelial-mesenchymal transformation and proliferation within the endocardial cushions. Furthermore, we demonstrate that abnormal epithelial-mesenchymal transformation can be reproduced in explant cultures derived from E10.5 mutant hearts, a time before neural crest cells migrate into the cardiac region. We provide evidence that neurofibromin normally regulates endocardial cushion formation by modulating ras activity. We suggest that analysis of the cardiac abnormality of Nf1-deficient mice may serve as a useful model for understanding normal and abnormal cardiac development and for the further analysis of neurofibromin function.

MATERIALS AND METHODS

Mice breeding and genotyping
Nf1+/− heterozygous mice (maintained on 129/Sv background) generated by gene targeting were generously provided by Dr Tyler Jacks (Jacks et al., 1994). Mice were bred at the animal care facility of the Children's Hospital of Pennsylvania with a 12/12 hour light-dark cycle. Noon of the day of the vaginal plug was considered day 0.5 of gestation (E0.5). The genotype of adult mice and embryos was confirmed by PCR of genomic DNA extracted from tail biopsies and extraembryonic membranes respectively, using previously described primers and PCR conditions (Jacks et al., 1994).

Histology
Isolated embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight followed by dehydration in an ethanol series, clearing in xylene and paraffin embedding in Paraplast plus. Eight to 10 μm sections were prepared on Superfrost slides and processed for routine hematoxylin and eosin staining or for in situ hybridization.

In situ hybridization
35S-labeled antisense riboprobes were synthesized by in vitro run-off transcription of linearized plasmids, using SP6, T7 or T3 RNA polymerase and 35S-UTP. Prehybridization, hybridization and posthybridization procedures were as described by Lutz et al. (1994). Slides were then dipped in Kodak NTB2 emulsion and exposed for 8–10 days at 4°C, developed and fixed in Kodak Dektol developer and fixer, counter stained in Hoechst 33258 (Sigma) for 2 minutes, washed in running water, dried and mounted in Canada Balsam. The Nf1 probe was prepared by PCR amplification of a 558 bp 3′ untranslated region fragment corresponding to positions 8542-9100 (GenBank accession no. L10370) which was cloned in pBlueScriptKS+ and named pKS-NIG. The construct was confirmed by direct sequencing. The insert contains a portion of the Nf1 transcript common to all known isoforms, pKS-NIG was linearized with HindIII and the riboprobe transcribed with T7 RNA polymerase. Sense control probe was prepared by linearizing with BamHI and transcribing with T3 RNA polymerase. The Msx2 probe corresponded to the 1.6 kb murine sequence (GenBank accession no. L11738). The PECAm (Baldwin et al., 1994), neurogenin (Meyer and Birchmeier, 1995), erbB3 (Meyer and Birchmeier, 1995), and Del1 (Hida et al., 1998) probes have been described previously.

Cell proliferation assay
Cell proliferation was assayed in situ in E12.5 embryos using 5-Bromo-2′-deoxy-uridine (BrdU). Pregnant mice were injected in the tail vein with 100 μg/gm body weight BrdU (Boehringer Mannheim) diluted in PBS, 1 hour prior to killing. Embryos were fixed and embedded as described above. Paraffin sections were deparaffinized in xylene, hydrated through an ethanol series and washed in PBS. Sections were denatured in 2N HCl for 30 minutes at room temperature, neutralized by washing in PBS, and incubated in blocking solution (1% BSA, 0.2% low fat milk and 0.3% Triton X-100) for 15 minutes at room temperature. Mouse monoclonal anti-BrdU antibody (Boehringer) diluted 1:100 in blocking solution was added and slides were incubated at 4°C for 12 hours. Slides were washed in PBS, incubated in Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch Labs) diluted 1:1000 in blocking solution at room temperature for 1 hour, washed in PBS, and mounted with Vectashield (Vector Laboratories).

Apoptosis (TUNEL) assay
Apoptotic cells were detected in situ on paraffin sections using a modified TUNEL technique (protocol kindly provided by E. Fernandez, University of Texas Southwestern) by specific end-labeling of fragmented nuclear DNA with biotin-dCTP (GIBCO/BRL). Slides were deparaffinized, hydrated, washed with PBS, treated with proteinase K (20 μg/ml PBS) for 10 minutes at room temperature, washed with PBS, permeabilized with 0.3% Triton X-100 in PBS for 10 minutes at room temperature, incubated in TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 5 minutes at room temperature and then in TdT enzyme mix containing biotin-dCTP (TdT buffer containing 20 ng/ml biotin-dCTP and 300 U/ml TdT enzyme) at 37°C for 90 minutes in a humidified chamber. Slides were then washed in PBS, pH 8.2 and biotin-labeled fragmented DNA was detected by Texas-Red labeled streptavidin (Vector laboratories) diluted 2:100 in PBS, pH 8.2 (30 minutes at room temperature) followed by final extensive washing in PBS and mounting in Vectashield (Vector Laboratories). DNase I-treated tissue was used as a positive control and equivalent slides without TdT enzyme were used as a negative control.

Collagen gel cultures
Type I rat collagen (Collaborative Research) gel (1 mg/ml) was prepared in 48-well plates according to the manufacturer’s recommendations. The formed gel was flushed and saturated with OptiMEM medium containing 1% fetal calf serum, ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium) (Collaborative Research) and antibiotics (50 μg/ml streptomycin, 50 μg/ml penicillin and 2.5 μg/ml fungizone). Endocardial cushion explants (including a small piece of adjacent myocardium) from wild-type and Nf1−/− E10.5 embryos were microdissected using tungsten needles under a dissecting microscope. Explants were placed on the drained collagen gel and incubated in 5% CO2 at 37°C in a humidified incubator.

Tissue was allowed to settle on the gel for 3–4 hours at which time 100 μl of fresh medium +/− virus (105 pfu) was added. After 12 hours,
medium was removed and replaced with fresh medium without virus. Cultures were examined and scored for gel invasion at 48-72 hours after explantation using inverted microscopy with Hoffman optics. Representative cultures were photographed with Kodak Ektachrome slide film and slides were scanned and processed in Adobe Photoshop. For conditioned media experiments, 5-10 primary explants were cultured in tissue culture dishes with 100 \( \mu l \) medium for 12 hours followed by extensive washes with fresh media and further culture for 24 or 48 hours. The medium was then used to culture fresh explants on collagen gels. AdLacZ adenovirus was kindly provided by Dr Jonathan Fox (University of Pennsylvania). ras-N17 and ras-61L adenovirus (Leone et al., 1997) were kindly provided by Dr J. R. Nevins (Duke University – HHMI).

RESULTS

Neurofibromin-deficient embryos exhibit abnormalities of outflow and atrioventricular endocardial cushions

Neurofibromin expression is widespread in the developing mouse embryo beginning at E8.0 (Huynh et al., 1994) and expression in the embryonic heart has been demonstrated by Northern blot and immunohistochemical analysis (Daston and Ratner, 1992; Huynh et al., 1994). More specific localization of \( Nf1 \) mRNA transcripts within the heart has not been reported. Using a riboprobe designed to detect all known isoforms of \( Nf1 \), we found ubiquitous expression at E10.5 (Fig. 1a) with a particularly strong signal in the dorsal root ganglia. Beginning at E11.5, we detected \( Nf1 \) expression in the endocardial cushions in the AV region (yellow arrow, Fig. 1b) and in the outflow tract (not shown). Endocardial cushion expression continued through E13.5 (Fig. 1c and d). A lower level of expression was also detected in the myocardium, and extensive expression was seen outside of the heart including the neural tube, the dorsal root ganglia and the limb mesenchyme (Fig. 1). Hence, \( Nf1 \) is expressed in endocardial cushions in addition to the known expression in myocardium of the developing heart.

Inactivation of the \( Nf1 \) gene results in midgestation cardiac failure (Brannan et al., 1994; Jacks et al., 1994). Our analysis of over 30 homozygous mutant \( Nf1 \) embryos and wild-type litter mates revealed a spectrum of developmental cardiac defects. In all cases, the mutant embryos displayed a dramatic overabundance of spongy appearing tissue in endocardial cushions of the outflow tract (compare Fig. 2a with 2b, yellow arrowheads indicate the endocardial cushions) and the atrioventricular region (compare Fig. 2c to 2d). The mutant cushions, unlike the wild-type controls, failed to condense and thin to form mature valve leaflets by E13.5. Double outlet right ventricle (DORV) was also invariably present. However, we never saw persistent truncus arteriosus; septated aortae and pulmonary arteries were discernible by E13.5. The degree of myocardial compact layer thinning (red arrowhead, Fig. 2d) was variable, as was the extent of venous and hepatic congestion. In the most severe examples, endocardial cushion

![Fig. 1.](image1.png) **Fig. 1.** \( Nf1 \) mRNA is expressed in the developing endocardial cushions. Radioactive in situ hybridization of E10.5-E13.5 embryos (transverse sections, dorsal at the top) reveals \( Nf1 \) mRNA expression within the endocardial cushions (yellow arrows) beginning at E11.5 (b) and continuing through E12.5 (c) and E13.5 (d). Expression was also evident diffusely at E10.5 (a) and in many tissues including neural tube (nt), dorsal root ganglia (drg) and limb (lb) at all time points tested. \( Nf1 \) is also weakly expressed in the heart (ht) myocardium.

![Fig. 2.](image2.png) **Fig. 2.** Enlarged endocardial cushion in \( Nf1^{--} \) hearts. Histology of E13.5 wild-type (a,c) and \( Nf1^{--} \) (b,d) hearts reveals a gross overabundance of tissue in the region of the outflow tract (a,b) and atrioventricular (c,d) endocardial cushions (ec, indicated by yellow arrows). The aorta (Ao) of the mutant heart (b) is partially compressed and a large pericardial effusion (*) is present. The aorta and pulmonary artery (PA) both arise from the right ventricle (rv) of the mutant heart producing double outlet right ventricle. Mature valve leaflets are not present in the mutant hearts. Note the dilated right atrium (RA) of the mutant heart and the thinned myocardium (red arrow in d). A ventricular septal defect (not shown) was also present. lv, left ventricle.
enlargement was so dramatic that inflow to the heart appeared to be compromised; right and left ventricles appeared hypoplastic and severe venous congestion and a persistent distended sinus venosus was present. No morphologic defect of cardiac development was noted in heterozygous embryos.

The endocardial cushions are normally populated by endocardial cells that develop into mesenchyme and invade the cushion matrix. This process is associated with alterations in gene expression including repression of endothelial-specific genes (such as PECAM and neuregulin) and activation of mesenchymal genes (such as PDGFαR, Del1, Msx2 and erbB3). We examined the expression of these genes in E13.5 Nf1−/− embryonic hearts and in wild-type litter mates (Fig. 3). All of the mesenchymal markers tested were expressed in the mutant endocardial cushions, although the expression of several of these (Del1, Msx2 and erbB3) appeared to be excluded from the central region of the bulky abnormal cushions (yellow arrows, Fig. 3). This was not the case for PDGFαR, which was expressed throughout both wild-type and mutant hearts. Sense probes gave no detectable signal.

Within the abnormal cushions have not completely repressed endothelial cell gene expression (e.g. neuregulin) and have not activated the full array of mesenchymal genes.

**Enhanced proliferation and decreased apoptosis within Nf1−/− endocardial cushions**

We analyzed the relative degrees of proliferation and cell death within mutant and wild-type endocardial cushions at E12.5 in order to elucidate the mechanism of cellular accumulation in the mutant hearts. As shown in Fig. 4a and c, very few proliferating cells were seen within the endocardial cushions of mutant hearts in Nf1−/− embryos. In wild-type embryos few actively dividing cells are noted within the AV cushion (yellow arrow in a, higher power shown in c). In Nf1−/− AV cushions, many BrdU-positive cells are seen (yellow arrow in b, higher power shown in d). lv, left ventricle, rv, right ventricle.
of wild-type embryos at this stage. By E12.5, epithelial-mesenchymal transformation had abated and the cushions began to thin out and form mature valvular structures. In contrast, analysis of mutant hearts at E12.5 revealed many proliferating cells throughout the A V cushions (Fig. 4b,d). Similar results were seen in the OT region (not shown).

Our analysis also indicated that apoptosis normally occurs within a specific region of the A V cushion at E12.5 (Fig. 5a,e). This is probably part of the normal process that allows for the reduction of cushion size and morphogenesis of the valves. TUNEL assays revealed a striking lack of apoptotic nuclei within the cushions of Nf1−/− hearts (b, higher power shown in f). False positive signal within the myocardium is due to autofluorescence of red blood cells, as evidenced by the strong signal detected from these cells under alternative filters. Images of the sections shown in a and b are taken with a FITC filter in c and d, respectively. Red blood cells appear green. Note the absence of red blood cells within the cushion region (yellow arrows). Lv, left ventricle; rv, right ventricle.

Enhanced epithelial-mesenchymal transformation by Nf1−/− explants

Invasion of the collagen gel by endocardial cushion explants derived from chick and mouse embryos has been used as a model of epithelial-mesenchymal transformation (Runyan and Markwald, 1983; Nakajima et al., 1997; Yamamura et al., 1997). We performed microdissections of E10.5 endocardial cushions from wild-type (a,c) or Nf1−/− mutant (b,d) embryos and culture for 72 hours. Transformed cells are elongated and can be seen to invade the collagen gel (arrows). Five to 15 cells per explant invade the gel from wild-type AV tissue (a) while greater than 50 cells invade from mutant tissue (b). Invasion of mutant cells is prevented by infection with a dominant negative ras adenovirus (ras-N17) (d). Viral infection of endothelial and invading cells is apparent after β-galactosidase staining of explants infected with adenovirus encoding lacZ (c). Infected cells are stained blue.
The marked difference from controls was evident as early as 24 hours after explantation. This was true if the explant was derived from the AV or OT regions, but no invasion was seen if explants were derived from the ventricular (non cushion) regions of either wild-type or mutant hearts.

**Ras activity is required for endocardial-mesenchymal transformation**

Since neurofibromin is capable of modulating ras activity by acting as a ras GAP, we sought to determine whether the enhanced epithelial-mesenchymal transformation that we observed in explant cultures could be inhibited by blocking ras signaling. We infected explant tissue with adenovirus encoding either lacZ (control) or dominant negative ras (ras-N17) (Leone et al., 1997) under conditions such that many of the endothelial cells were infected, as determined by β-galactosidase expression and staining (Fig. 6c). In every case, the dominant negative ras virus was able to completely prevent or significantly impair mesenchymal cell invasion by mutant explants (Fig. 6d). The tissue remained viable as evidenced by the continued contractions of myocardium in culture. Infection with the control lacZ virus did not prevent invasion. An intact ras signaling pathway is therefore necessary for the abnormal Nf1−/− endothelial-mesenchymal transformation phenotype in explant cultures.

**Ras activation is sufficient to enhance endocardial-mesenchymal transformation and to mimic the Nf1−/− mutant phenotype**

We infected wild-type endocardial cushion explants with adenovirus encoding lacZ (control), dominant negative ras (ras-N17) (Leone et al., 1997), or constitutively activated ras (ras-61L) (Leone et al., 1997). Dominant negative ras virus prevented the low level of invasion seen in wild-type explants (Fig. 7a,b) suggesting that ras signaling is required for normal endocardial-mesenchymal transformation as well as for the enhanced transformation seen in the Nf1 mutant explants. As shown in Fig. 7c-g, activated ras infection resulted in a dramatic increase in the number of cells invading the gel and the degree of migration compared to lacZ controls. The explants infected with the activated ras virus resembled the Nf1 mutant explants. Thus, activated ras signaling is both necessary (Fig. 6) and sufficient (Fig. 7) for endocardial-mesenchymal transformation.

Nf1 is expressed in both the myocardium overlying the endocardial cushions, and within the cushions themselves. Myocardium is required for endothelial-mesenchymal transformation within the cushions, and secretes factors, including TGFβ, that mediate this process (Potts and Runyan, 1989; Ramsdell and Markwald, 1997). Therefore, we tested the hypothesis that enhanced ras activation in the myocardium resulted in augmented secretion of a substance responsible for enhancing endothelial-mesenchymal transformation. First, we cocultured wild-type explants with whole explants (n=11) or with AV myocardium (n=22) that had been previously treated with the activated ras adenovirus or with control lacZ adenovirus. No difference in the invasive properties of the cocultured wild-type explant was noted suggesting that ras-treated explants do not secrete a factor able to influence transformation. Next, we tested the effect of conditioned medium after incubation for 24 or 48 hours with multiple ras infected explants or lacZ infected controls (n=12 each time). No effect of conditioned medium was observed. Finally, we tested the ability of isolated wild-type, mutant or ras infected endocardium to invade a collagen gel in the absence of associated myocardium. In no case was wild-type or mutant

Fig. 7. Activation of ras signaling enhances endothelial-mesenchymal transformation. E10.5 wild-type AV cushion explants were infected with control lacZ adenovirus (a) and stained for β-galactosidase. Infected cells (arrow) are blue. Parallel cultures were infected with dominant negative ras adenovirus (ras-N17: b) or constitutively activated ras adenovirus (ras-61L: c-g) for 12 hours and imaged after 72 hours. Dominant negative ras inhibited cellular invasion of the collagen gel, while constitutively activated ras augmented invasion and resulted in a morphological appearance very similar to that of the Nf1−/− explants (compare Figs 7c with 6b). A series of photographs of another Nf1−/− explant infected with ras-61L were taken at varying focal planes (d-g) to clearly demonstrate invasion of cells into the collagen gel.
are most consistent with a cell autonomous role for induction of apoptosis in the endocardial cushions. Our data and proliferation and to allow for the appropriate temporal required to modulate epithelial-mesenchymal transformation

valves. The molecular signals governing this transition from occurs and the bulky cushions are remodeled into thin cardiac differentiation and morphogenesis of mesenchymal structures.

invasion and proliferation. Downregulation of the intracellular factor activation of a receptor tyrosine kinase on endothelial cells activates an intracellular ras-mediated signaling cascade and contributes to epithelial-mesenchymal transformation, invasion and proliferation. Downregulation of the intracellular ras-transduced signal is mediated by the GAP activity of neurofibromin which is expressed by endothelial cells that enter the cardiac jelly. Terminal differentiation into mature cardiac valvar structures ensues associated with the concomitant expression of mature mesenchymal markers, while a subgroup of the mesenchymal cells undergo apoptosis lessening the bulk of the cushion tissue (Lamers et al., 1995 and this report).

This model is supported by genetic and experimental data. First, a series of explant experiments performed with chick endocardial cushion tissue has demonstrated the requirement for myocardial-derived factors to trigger endothelial transformation. These factors can be partially replaced by TGFβ3 (Potts and Runyan, 1989; Rasmussell and Markwald, 1997), and antibodies to the TGFβII receptor can block transformation and invasion (Brown et al., 1996). The TGFβII receptor can signal via several intracellular pathways, including ras (reviewed in Kolodziejczyk and Hall, 1996).

Another candidate receptor tyrosine kinase expressed in the transformed cushion mesenchyme is PDGFRαR. Homodimers of PDGFRαR can bind homodimers of the secreted ligand PDGF-A which is expressed at high levels in the myocardium of E9.5-E11.5 mice (our unpublished observations). Deletion of PDGFRαR in the Patch mouse is associated with improper development of the endocardial cushions (Schatteman et al., 1995), though these defects were not reported for the more precise PDGFRαR knock-out recently described (Soriano, 1997).

The association between erbB3 expression and the phenotype we observe in the Nf1−/− mouse is of particular interest because of the emerging role of the neuregulin/erb pathway in Schwann cells. Schwann cells are the predominant cell type in neurofibromas associated with neurofibromatosis and are markedly abnormal in Nf1−/− mice (Kim et al., 1997; Kim et al., 1995). We have noted enlarged dorsal root ganglia in several Nf1−/− embryos with enhanced expression of erbB3 and neuregulin by in situ hybridization (unpublished observations), and the coexpression of this receptor and ligand in Schwann cells has been postulated to induce a ras-mediated autocrine loop (Rosenbaum et al., 1997). In addition, neuregulin stimulation of Erb receptors has been strongly implicated in modulation of Schwann cell apoptosis, a pathway that might also be mimicked in the endocardial cushion (Grinspan et al., 1996; Syroid et al., 1996). Interestingly, all of the endocardial cushion mesenchymal markers that we tested (PDGFRαR, Msx2, Del1, erbB3) were also expressed in migrating neural crest cells or the dorsal root ganglia (or both) suggesting a similarity in gene expression programs between these tissues and the endocardial cushions.

Neurofibromatosis affects multiple neural crest lineages, including Schwann cells and melanocytes. The known contribution of neural crest to the developing heart originally suggested an appealing explanation for the cardiac phenotype of neurofibromin-deficient mice (Brannan et al., 1994; Jacks et al., 1994). Neural crest is required for proper morphogenesis of the outflow tract including septation of the single great vessel emerging from the primitive heart into the aorta and the pulmonary artery (Kirby et al., 1983). Our analysis indicates that the endocardial cushion hyperplasia in Nf1−/−embryos is not a neural crest disorder. This abnormality clearly involves the AV as well as the OT cushions, and neural crest cells have not been demonstrated to migrate as far as the AV cushion region (Noden et al., 1995). Furthermore, explants of mutant cushion tissue taken prior to the time that neural crest cells reach the heart region (Conway et al., 1997) display abnormal characteristics in culture including enhanced invasion of a collagen gel matrix. The endocardial cushion defect is due to enhanced endothelial-mesenchymal transformation. Our in situ analysis indicates that the transformed cells continue to proliferate within the cushion at a time when wild-type mesenchymal cells are differentiating or undergoing apoptosis.

Although we cannot rule out the possibility that double outlet right ventricle observed in the mutant hearts is due to a second and unrelated defect of cardiac neural crest, we suspect that this morphogenetic defect is secondary to the abnormal proliferation of mesenchymal cells in the OT cushions. We propose that double outlet right ventricle must not in all cases
be related to a neural crest abnormality, but rather is related to abnormal OT cushion remodeling which in turn can be caused by neural crest or non-neural crest defects. In accord with this model, we never observed any degree of persistent truncus arteriosus or an aortopulmonary window suggestive of defective outflow tract septation and a clear sign of neural crest deficiency, though the phenotype of double outlet right ventricle was completely penetrant.

In humans, type I neurofibromatosis is not clearly associated with congenital heart disease. This may be due to a species difference, but more likely is related to the fact that NF1 patients inherit only one mutated allele of the \textit{NF1} gene, and presumably acquire a ‘second hit’ in the remaining wild-type allele in a somatic tissue leading to tumor formation. We would hypothesize that a ‘second hit’ would have to occur in an endocardial cell progenitor during a limited period of early or mid gestation in order to induce congenital heart disease. Homozygous NF1 germline mutation or a very early gestation ‘second hit’ would likely be embryonic lethal. However, case reports of patients with congenital heart disease and Watson or Noonan syndrome have documented NF1 mutations (Abuelo and Meryash, 1988; Tassabehji et al., 1993), and these syndromes are associated with cardiac defects related to defective endocardial cushion development.

The mechanism by which neurofibromin modulates transformation and proliferation in the heart is of significant interest since it may relate directly to the role of NF1 as a tumor suppressor gene. Our explant culture experiments suggest that, in this tissue, neurofibromin acts to modulate ras mediated signaling. Forced activation of ras can mimic the mutant culture characteristics and inhibition of ras can rescue the mutant phenotype. Neurofibromin contains a GAP related domain, and increased ras activity has been documented in neural and hematopoietic tissue from NF patients and animals (Bernards, 1995; Bollag et al., 1996; Guha et al., 1996; Kim, H. A., Rosenbaum, T., Marchionni, M. A., Ratner, N., Nordlund, M. I., Reid, S. W., Buchberg, A. M., Jenkins, N. A., Parada, L. F. and Copeland, N. G. (1994). Targeted disruption of the neurofibromatosis type-1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. Genes Dev. 8, 1019-1029.


REFERENCES


