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NRP1 haploinsufficiency predisposes to the development of Tetralogy of Fallot

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Abstract

Tetralogy of Fallot (TOF) is the most common cyanotic congenital heart defect. It involves anatomical abnormalities that change the normal flow of blood through the heart resulting in low oxygenation. Although not all of the underlying causes of TOF are completely understood, the disease has been associated with varying genetic etiologies including chromosomal abnormalities and Mendelian disorders, but can also occur as an isolated defect. In this report, we describe a familial case of TOF associated with a 1.8 Mb deletion of chromosome 10p11. Among the three genes in the region one is *Neuropilin1 (NRP1)*, a membrane co-receptor of *VEGF* that modulates vasculogenesis. Hemizygous levels of *NRP1* resulted in a reduced expression at the transcriptional and protein levels in patient-derived cells. Reduction of NRP1 also lead to decreased function of its activity as a co-receptor in intermolecular VEGF signaling. These findings support that diminished levels of NRP1 contribute to the development of TOF, likely through its function in mediating VEGF signal and vasculogenesis.

Keywords

chromosomal deletion; congenital heart disease; neuropilin 1 (NRP1); prenatal ultrasound; tetralogy of fallot (TOF)

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CONFLICTS OF INTEREST

None.

1 INTRODUCTION

Congenital heart disease (CHD) affects approximately 1% of live births and is the most common life-threatening birth defect in the United States. Among these congenital conditions are conotruncal heart defects (CTDs) that consist of structural abnormalities of the outflow tracts of the heart, and account for 20–30% of all CHD (Hobbs et al., 2014). The three major types of CTDs include truncus arteriosus, transposition of the great arteries, and Tetralogy of Fallot (TOF). TOF is the most common cyanotic congenital heart defect, occurring in 1 per 2,518 live births (Parker, Abt, & Parr, 1980). The classic form of TOF consists of four anatomic defects: ventricular septal defect (VSD), obstruction of the right ventricular outflow tract (pulmonary stenosis), overriding aorta and right ventricular hypertrophy (Dabbagh, Conte, & Lubin, 2017).

Historically, TOF was considered a disorder of multigenic etiology. However, population studies have demonstrated a recurrence risk ratio of 11.7 for conotruncal defects (Øyen et al., 2009), suggesting that Mendelian inheritance may account for a portion of the cases. A comprehensive genotype-phenotype analysis in 230 patients with TOF showed that it is often part of the constellation of findings in many genetic syndromes including Mendelian disorders and aneuploidy (Rauch et al., 2010). At this time, the most common identifiable cause of TOF is chromosome 22q11 deletion syndrome, a relatively common aneuploidy characterized by congenital heart disease, palatal abnormalities, hypocalcemia, characteristic facial features, learning difficulties, and immunodeficiency (Burn et al., 1993; Driscoll et al., 1993; Momma, Kondo, Ando, Matsuoka, & Takao, 1995). Supporting chromosome 22q11 as TOF susceptibility region, TOF has also been reported in rare cases of 22q11 microduplication syndrome (Hu et al., 2011). The second most common currently identifiable cause of TOF is down syndrome due to trisomy for chromosome 21 (OMIM 190685) (Baraona, Gurvitz, Landzberg, & Opotowsky, 2013; CASE RECORDS of the Massachusetts General Hospital, 1957; Ghosh & Biswas, 1955), followed by other chromosomal aberrations and copy number changes (Greenway et al., 2009; Lammer et al., 2009; Rauch et al., 2010; Silversides et al., 2012). A lesser percentage of TOF results from its occurrence in Mendelian disorders. For example, it is the characteristic congenital heart defect in Alagille (OMIM 118450) and Kabuki (OMIM 147920) syndromes, due to heterozygosity for mutations in *JAG* or *NOTCH2* (OMIM 118450, OMIM 610205) and *KMT2D* (OMIM 147920), respectively (Krantz et al., 1999; McDaniell et al., 2006; Ng et al., 2010; Rauch et al., 2010). Although many TOF cases are associated with phenotypically complex syndromes, TOF can occur as an isolated non-syndromic defect. However, only a small percentage of isolated TOF cases have a recognized genetic etiology and result from heterozygosity for mutations in the genes *TBX1* (MIM 602054), *JAG1* (MIM 601920), *NKX2.5* (MIM 600584), *ZFPM2* (MIM 603693), *TBX5* (MIM 601620), *GATA6* (MIM 601656), and *GATA4* (MIM 600576) (Benson et al., 1999; Burn et al., 1998; Eldadah et al., 2001; Maitra, Koenig, Srivastava, & Garg, 2010; Pizzuti et al., 2003; Rauch et al., 2010; Schott et al., 1998; Tomita-Mitchell, Maslen, Morris, Garg, & Goldmuntz, 2007).

Genome-wide association studies (GWAS) have been employed to identify risk alleles associated with TOF and have suggested a susceptibility locus on chromosome 10p11 (Cordell et al., 2013; Xu et al., 2014), however no specific gene in the region is yet shown to

be causative. We identified a fetus and his affected father who both had TOF and harbored a chromosomal 10p11.22-p11.21 deletion. Within the region are three genes, including *Neuropilin 1, NRP1* (MIM 602069). NRP1 is a receptor for semaphorins and vascular endothelial growth factor (VEGF). It has been demonstrated to function as a co-receptor of VEGFR2 to modulate cardiovascular development and vasculogenesis (Lampropoulou & Ruhrberg, 2014; Lee et al., 2002). Affected patient derived cells show diminished levels of NRP1 cDNA and protein, misregulation of VEGF signaling, and support a novel association between defective NRP1 levels and the development of TOF.

2 MATERIALS AND METHODS

2.1 Clinical information

Patients and their family members were assessed under a University of California at Los Angeles approved human subjects protocol. Clinical information and imaging studies were reviewed to determine the phenotypic findings in the affected individuals. Amniocytes were collected from proband R14-031A. No parental cells were available for this study.

2.2 SNP array

SNP microarray analysis was performed using the Affymetrix Cytoscan HD platform. DNA was processed and hybridized to the Affymetrix Cytoscan HD GeneChip. Data was analyzed using Chromosome Analysis Suite

2.3 Sequence analyses and epigenetic studies

DNA was isolated from blood or cultured fibroblasts via a standard manufacturer's protocol (QIAGEN, Hilden, Germany). Sanger sequence analysis of the remaining hemizygous allele of *NRP1* in the proband was performed. Whole cDNA *NRP1* sequence derived from amniocyte RNA was analyzed to identify other potential transcriptional defects. Complete sequencing of cDNA, including the 5′ UTR to account for possible non-coding variants in family members, was performed from blood DNA. Bi-allelic expression was studied by sequencing human *NRP1* cDNA from amniocytes, endothelial cells, and striated muscle searching for heterozygosity for several high frequency variants (Ensembl.org: rs2229935, 0.35 MAF frequency; rs2229934, 0.35 MAF frequency; COSM146924 0.5 MAF frequency). cDNA derived from mouse heart was sequenced to identify polymorphisms between strains C57BL/6J and C3H/HeJ (*Nrp1* SNP352). Primers are shown in Supplementary Table S1.

2.4 Quantitative expression analysis and genomic DNA

Total RNA was extracted from cultured amniocytes at approximately 80% confluence using TRIzol (Invitrogen, Carlsbad, CA), treated with DNAase (Invitrogen) and underwent reverse transcription (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Quantitative PCR was performed with a real-time PCR detection system (Stratagene, La Jolla, CA, MX3005P) using SYBR green PCR master mix (Thermo Scientific) and optimized thermocycler conditions. qPCR was performed in three independent experiments. Each sample was analyzed using two housekeeping genes to normalize for RNA/cDNA input amounts and to determine relative quantifications. Levels of transcripts in controls were set at 1. Melting curve analysis showed a single, sharp peak with

the expected temperature melting for all samples. DNA quantitative PCR was also analyzed with primers within and outside the deletion region to assess potential hemizygosity in other family members. Primers were designed with pairs from multiple different exons (Supplementary Table S1).

2.5 Western blot

For Western blot analyses, proteins lysates were separated by electrophoresis through a 10% SDS-polyacrylamide gel, transferred to PVDF membranes, blocked in 5% milk and probed with primary antibodies (anti-NRP1 antibody, 1:2000 Proteintech, Chicago, IL; anti-GAPDH 1:2000 Cell Signaling, Danvers, MA, 2118S). Peroxidase-conjugated secondary antibodies (Cell Signaling 7071 and 7072) were used and immuno-complexes identified by using the ECL detection reagent (Cell Signaling 7003). FIJI was used to quantify bands following Gel Analysis recommendations from ImageJ (Gassmann, Grenacher, Rohde, & Vogel, 2009) and the Mann–Whitney test was performed for statistical analysis using Prism software. Experiments were replicated three times.

2.6 Amniocytes and endothelial cells co-culture

Human umbilical vein endothelial cells (EC) (100,000 cells) were cultured alone or with control versus patient amniocytes (100,000 cells) in MCDB-131 with 10% FBS for at least 48 hr. The cells were incubated in media without serum overnight, treated with 200 μM Na_3VO_4 in MCDB-131 for 5 min at 37 °C, then VEGF (100 ng/ml) in MCDB 131 for 5 min 37 °C. Cells were washed with PBS then lysed in modified RIPA (mRIPA) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycolate, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, and protease inhibitor cocktail (Roche, Pleasanton, CA). Lysates were agitated at 4 °C for 30 min, then centrifuged for 10 min at 4 °C to remove debris. Proteins were separated by SDS-PAGE and immunoblot analyses were performed with antibodies against total VEGFR2 and phospho-VEGFR2 Y1175 (1:1000 Cell Signaling), NRP1 (1:2000 Proteintech), smooth muscle actin (SMA, an amniocyte marker, 1:1000 Cell Signaling), and GAPDH as a loading control (1:1000 Cell Signaling). Densitomery analysis was performed using ImageLab software (Bio-Rad) and the signal of phospho-VEGFR2 relative to total VEGFR2 was determined. This experiment was performed one time because the patient's amniocytes failed additional passages.

3 RESULTS

3.1 Clinical findings

The proband, patient reference number R14-031A was the offspring of a 32 year-old G2P1 mother and 37 year-old father. Fetal ultrasound at 16 weeks showed findings consistent with TOF that included narrowing of the pulmonic vessel at the level of the pulmonic valve and a ventral septal defect (VSD). No abnormalities were detected in other organ systems. Continued fetal evaluation by fetal echocardiography showed at 26 weeks moderate valvular/subvalvular pulmonary stenosis and moderately hypoplastic pulmonary arteries as well as a double aortic arch (Figure 1a). Postnatal echocardiogram showed the aforementioned anomalies and an overriding aorta (Figure 1b). At birth the child had no

other appreciable abnormalities. The patient underwent surgical correction of the TOF at the age of 2 months.

On further questioning of the family at the time of the proband's 16 week fetal ultrasound the familial history revealed that the patient's father fatigued easily as a child and workup at age 4 years identified TOF and he underwent an uncomplicated surgical correction. The father also had unilateral equinovarus at birth that responded to physical manipulations and surgical correction was not necessary. As an adult, a horse-shoe kidney and fused thoracic vertebrae were noted by MRI. The father also reported unilateral congenital deafness. The patient's paternal uncle (Figure 2, II-3) has unilateral renal agenesis and there are two healthy paternal aunts (Figure 2, II-1 and 2). The remainder of the paternal and maternal family history was negative for cardiac defects, renal anomalies, skeletal malformations, or congenital deafness.

3.2 Genetic analyses

Because of the identification of the fetal TOF, amniocentesis was performed at 16 weeks (Figure 2a, III-3). SNP chromosomal microarray analysis from amniocyte derived DNA showed a 1.81 MB interstitial deletion at 10p11.22-p11.21 with estimated breakpoints at 32,686,908 to 34,498,716 base pairs (GRCh38/hg38: chr10:32686908–34498716). No other chromosomal abnormalities or rearrangements were detected by karyotype or microarray. Microarray analyses from DNA derived from peripheral blood in the parents revealed the same deletion in the proband's father (Figure 2a, II-4) and no abnormalities were noted in this region in the maternal sample (Figure 2a, II-5). FISH analysis was performed on blood samples from the paternal grandparents, revealed that the paternal grandfather also carried the deletion (Figure 2a, I-2). Further evaluation of the paternal family showed that the paternal uncle with the unilateral renal agenesis did not carry the deletion (Figure 2, II-3), though one unaffected aunt did (Figure 2, II-2). There are three genes localized in this region: *ITGB1* (MIM135630), *NRP1* (MIM 602069), and *PARD3* (MIM 606745) (Figure 2b).

3.3 Deletion results in reduced expression of NRP1

As the deleted region contains three genes, *ITGB1*, *NRP1*, and *PARD3*, we determined the effects of the deletion on these loci by studying the expression of the three genes by quantitative RT-PCR on amniocytes derived from the patient R14-031A (Figure 2a, III-3). Among the three genes, only *NRP1* showed reduced transcriptional levels, while *ITGB1* and *PARD3* cDNA levels were normal when compared with control amniocytes (Figure 3a-c). Interestingly, *NRP1* expression was below the predicted 0.5-fold compared to control cells, suggesting perhaps an additional factor reducing its expression other than the deletion. Data obtained from DECIPHER and ExAC databases indicate intolerance of *NRP1* to loss of function (pLi = 1.00) and low Haploinsufficiency score (HI index = 2.53%), suggesting that haploinsufficiency for *NRP1* may produce pathogenicity. To determine if transcriptional changes corresponded with reduced levels of proteins we performed Western blot analysis. These results showed that similar to cDNA levels, there was markedly decreased NRP1 protein in amniocytes from patient, compared to control (Figure 3d), lending to the

conclusion that *NRP1* haploinsufficiency due to the deletion contributed to reduced levels of NRP1 protein in patient derived amniocytes.

We then asked whether there was a sequence alteration on the maternally inherited *NRP1* allele that contributed to reduced cDNA and protein levels below the 50% predicted for haploinsufficiency, as well as perhaps uncovering a recessive mode of inheritance leading to TOF. Sanger sequence analysis of the coding exons of *NPR1* on the remaining maternal allele from affected amniocytes did not reveal any coding changes or silent polymorphisms. Furthermore, we asked if *NRP1* could be an imprinted gene or that other epigenetic changes that could account for marked reduced expression contributing to the phenotype. No database showed *NRP1* as an imprinting gene. We also performed cDNA sequencing to check for bi-allelic expression of three common synonymous variants in the *NRP1* coding sequence (Ensembl.org: rs2229935, 0.35 MAF frequency; rs2229934, 0.35 MAF frequency; COSM146924 0.5 MAF frequency) in amniocytes, endothelial cell lines and striated muscle derived from four control individuals. For each individual and in multiple tissues, there was bi-allelic expression of these variants (Figure 3e, rs2229935), discarding the possibility that *NRP1* is maternally imprinted. Due to the lack of human cardiac samples we could not test potential epigenetic changes in the heart, however, we were able to analyze the expression of *Nrp1* in mouse heart. Mouse *Nrp1* coding sequence contains a polymorphism variant between strains C57BL/6J and C3H/HeJ. RNA sequencing from heart samples of C57BL/6J-C3H/HeJ heterozygous males and females showed biallelic expression of the variant, suggesting no significant difference in allelic expression of *Nrp1* in mouse heart. (Figure 3f, SNP352). These studies suggest that the phenotype was not recessive due to haploinsufficiency plus a nonsilent sequence change, or through loss of *NRP1* through an epigenetic mechanism.

3.4 Decreased NRPlevels correlate with decreased NRP1 function

We then asked if reduced NRP1 protein levels observed in the patient amniocytes altered NRP1 function. NRP1 can modulate signaling by VEGFR2 by acting as a co-receptor for VEGF. While NRP1 by itself does not directly signal in response to VEGF, it alters the responsiveness of VEGFR2 to VEGF ligand. It has been reported that NRP1 on non-endothelial cells can be presented to VEGFR2 on endothelial cells in trans and suppress angiogenesis by reducing VEGFR2 internalization and signaling (Koch et al., 2014). We tested whether reduced expression of NRP1 on the patient amniocytes resulted in differences in trans signaling to VEGFR2. After co-culturing amniocytes with endothelial cells for 2 days, we challenged the cells with VEGF and assayed VEGFR2 signaling by measuring the phosphorylation of VEGFR2 relative to total receptor levels. We observed a 3.4-fold change when endothelial cells were co-cultured with patient amniocytes, while co-culture with control amniocytes had minimal effect by comparison (Figure 4), suggesting together with information from published findings that reduced the NRP1 levels resulted in less NRP1-VEGFR2 complexes in trans and loss of inhibition of VEGFR2 phosphorylation. Unfortunately, limited material did not allow for further studies. These findings suggest that increased VEGFR2 signaling due to reduced NRP1 inhibition may contribute to the phenotype through dysregulation of angiogenesis at a critical stage of development.

4 DISCUSSION

NRP1 is a transmembrane protein, which serves as a co-receptor for both vascular endothelial growth factor (VEGF; 192240) and semaphorin (SEMA3A; 603961) family members (Fantin et al., 2014; Lee et al., 2002). NRP1 plays a role in angiogenesis, axon guidance, cell survival, migration, and invasion. It is expressed in the digestive tract, urinary tract, adrenal glands, cardiac muscle, and CNS. A zebrafish knockout model demonstrated that NRP1 regulated angiogenesis through a VEGF dependent pathway (Lee et al., 2002). Knock-out mice for *Nrp1* die during development due to severe cardiovascular defects (Kawasaki et al., 1999; Kitsukawa et al., 1997). Targeted knockout mice have shown abnormalities of the cardiovascular system including persistent truncus arteriosus and abnormal vestibulocochlear development (Gu et al., 2003). Endothelial disruption of *Nrp1* in mice caused a DiGeorge syndrome-like phenotype that included septal defects (Zhou, Pashmforoush, & Sucov, 2012). Additionally, a genetic variant at 10p11 has been associated with increased risk of TOF in a Chinese population study (Xu et al., 2014). Specifically, Xu et al. suggested that the presence of a variant in the *NRP1* coding sequence (rs2228638) increases the risk of developing TOF. In a large scale study that included syndromic and nonsyndromic congenital heart defects, Sifrim et al, found a de novo rare variant in *NRP1*, but no specific details were reported (Sifrim et al., 2016). Finally, Shaheen, Hashem, and Alkuraya (2015), reported on a consanguineous family with three affected individuals that presented with recurrent truncus arteriosus due to homozygosity for a truncating splicing mutation in *NRP1* (p.Asp25Gfs*25) (Shaheen et al., 2015). These findings support *NRP1*'s association with cardiac defects, including TOF, making *NRP1* the most likely candidate to cause the heart defects in patients with 10p11 deletion syndrome.

The family case presented in this study showed that two of four members of the family with the 10p11 deletion had TOF (Figure 2), suggesting that deletion of this region at 10p11 has incomplete penetrance for the risk of developing TOF. Incomplete penetrance for TOF has been reported for chromosome 5q33 deletion syndrome (Starkovich, Lalani, Mercer, & Scott, 2016), GATA4 missense mutations (Chen et al., 2016), low frequency variants in ROCK1 (Palomino et al., 2013), and JAG1 (Eldadah et al., 2001). The genetic or non-genetic etiologies that contribute to incomplete penetrance remain unresolved. In this work, other potential mechanisms were addressed. No coding variations were detected in the remaining *NRP1* maternally inherited allele. Imprinting of the *NRP1* gene was discarded because two brothers with the deletion showed both absence and presence of TOF. Additionally, several control human cell lines showed biallelic *NRP1* expression, suggesting that alleles are not selectively silenced. One study showed that the *NRP1* had a lower methylation level in myocardial tissue in patients with TOF (Sheng et al., 2014) suggesting that methylation state of *NRP1* can be responsible for the cardiac defect, however, neither that study nor our results had evidence for altered *NRP1* expression levels. Our analysis included human amniocytes, endothelial cells, muscle, and murine heart, however, we cannot rule out a very localized epigenetic control in the development of the cardiac outflow tract at the arterial pole of the heart (Buckingham, Meilhac, & Zaffran, 2005) that could affect development. Other genetic changes that could explain the incomplete penetrance of the *NRP1* deletion are undetected variants in the remaining *NRP1* promoter, non-coding

changes affecting *NRP1* regulatory elements or digenic variant influences where more than one gene could be involved in the control of *NRP1* expression influencing development. Interestingly, the levels of *NRP1* cDNA and protein derived from affected amniocytes showed levels that were more than 50% reduced, the amount expected based on the deletion, perhaps suggesting that the regulation of *NRP1* is more complex than previously appreciated. Two other genes were deleted in the region, *ITGB1* and *PARP3*. Both of these genes have roles in development (Hirose et al., 2006; Wu et al., 2009), however mouse knockout models did not manifest with congenital heart disease and neither gene showed decreased levels of cDNA expression in affected amniocytes. Thus, *NRP1* remains the most likely candidate gene in the 10p11 deletion to contribute to TOF.

NRP1 was first discovered in 1987 as a cell adhesion molecule in xenopus neurogenesis (Takagi, Tsuji, Amagai, Takamatsu, & Fujisawa, 1987). It serves as a receptor for a class of semaphorin (SEMA3), a secreted ligand that regulates axon guidance (He & Tessier-Lavigne, 1997; Kolodkin & Ginty, 1997), and well as a receptor for specific isoforms of vascular endothelial growth factor A (VEGF) (Soker, Fidder, Neufeld, & Klagsbrun, 1996; Soker, Takashima, Miao, Neufeld, & Klagsbrun, 1998). Additionally, NRP1 can modulate PDGF, TGFβ, and integrin signaling (Cao et al., 2010; Evans et al., 2011; Pellet-Many et al., 2011; Valdembri et al., 2009; Yaqoob et al., 2012), thus the tissue dependent roles of NRP1 are complex. For example, conditional ablation of *NRP1* in endothelial cells caused cardiovascular development defects, while directed mutagenesis for *NRP1* semaphorin binding sites only showed defective neuronal development, but not cardiovascular abnormalities. (Gu et al., 2003; Vieira, Schwarz, & Ruhrberg, 2007). VEGF ligand binding induces complex formation between NRP1 and VEGFR2 enhancing VEGFR2 signaling during endothelial cell migration in vitro (Evans et al., 2011; Soker, Miao, Nomi, Takashima, & Klagsbrun, 2002; Wang, Zeng, Wang, Soker, & Mukhopadhyay, 2003) and as well as arteriogenesis in vivo (Lanahan et al., 2013). VEGF is a known modifier for TOF as low VEGF expression haplotypes have been observed to increase risk of non-syndromic TOF (Lambrechts et al., 2005), however, although the role of NRP1-VEGF in cardiovascular defects while well described in animal models, its role in human cardiac defects is poorly understood. Our data demonstrated that decreased NRP1 due to the deletion presented in trans from amniocytes to endothelial cells abnormally increased the internalization of the NRP1-VEGF-VEGFR demonstrating a functional role for the 10p11 deletion. In conclusion, our data shows that chromosome 10p11 deletion can produce TOF with incomplete penetrance and negative impacts VEGF signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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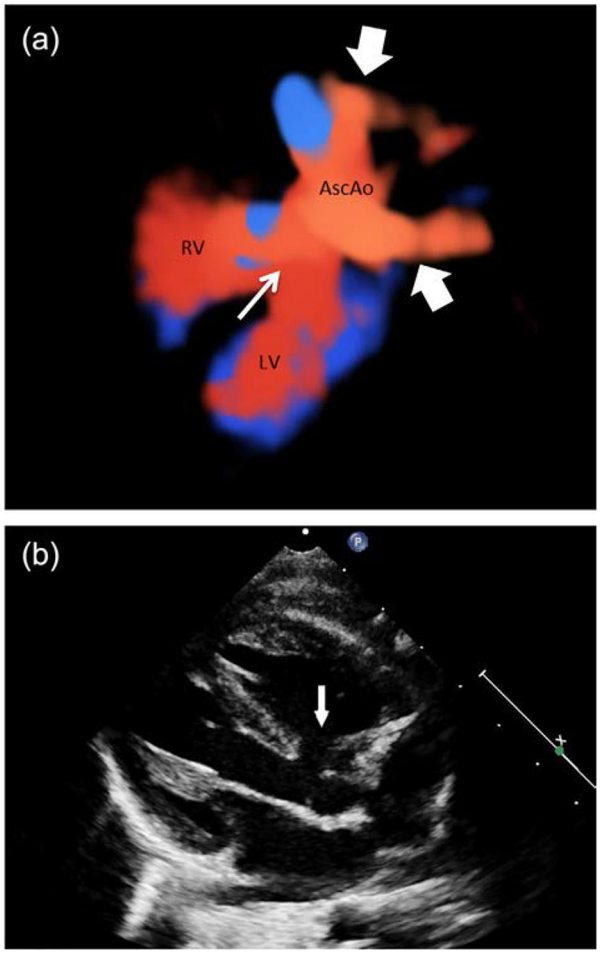

FIGURE 1.

Cardiac defects in patient R14-031A. (a) 3D rendering of fetal heart showing VSD (thin arrow) and double aortic arch (thick arrows). RV = Right ventricle. LV = Left ventricle. Asc Ao = Ascending aorta. (b) Postnatal ECHO showing VSD and overriding aorta (Arrow).

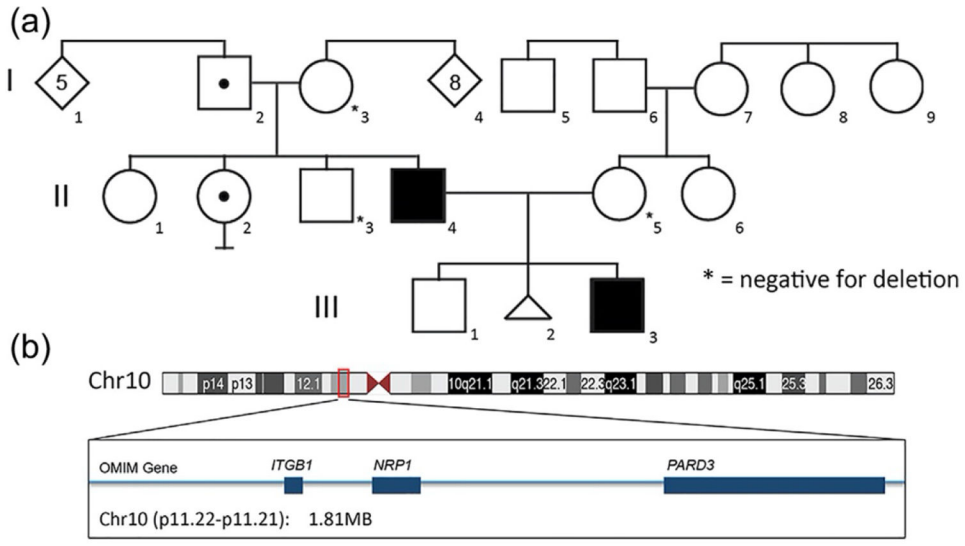

FIGURE 2.

Incomplete penetrance of the deletion 10p11. (a) Pedigree of R14-031 family with TOF patients (filled in black), carriers for the deletion 10p11 without TOF (dot inside symbol). Other members of the family were tested for the 10p11 deletion and were negative (*). (b) Diagram of the chromosome 10 with details of the deleted region 10p11.22-p11.21 including the genes *ITGB1*, *NRP1*, and *PARD3*.

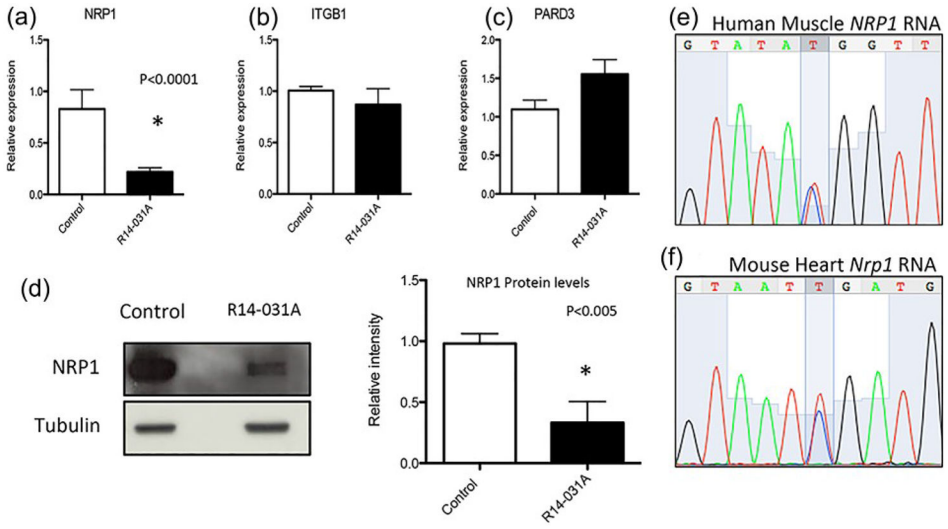

FIGURE 3.

Transcriptional levels for the three genes found in the deletion 10p11. (a) Expression levels of NRP1 in amniocytes from the patient R14-031A showed a significant decreased compared to the control. ITGB1 and PARD3 expression (b,c) did not show changes due to the deletion 10p11. (d) Protein levels on NRP1 were decreased in R14-031A amniocytes more than 0.5-fold. Reduction under 50% of NRP1 levels did not correspond with epigenetic changes as shown by bi-allelic expression of the NPR1 mRNA in human and mouse tissues (e,f).

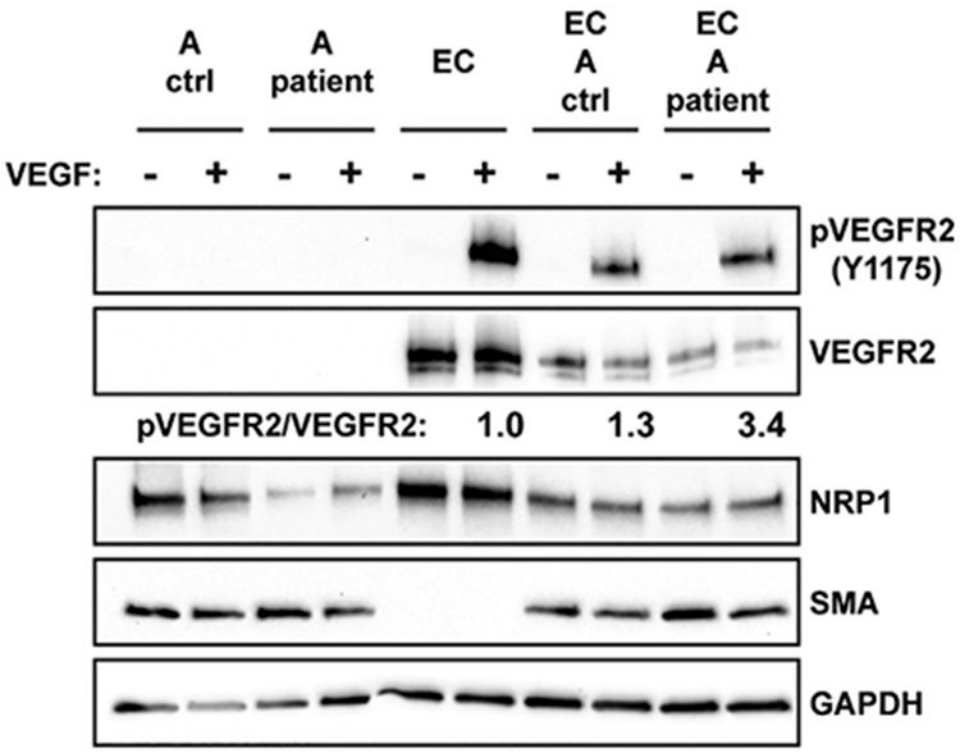

FIGURE 4.

Altered levels of VEGFR2 phosphorylation from patient amniocytes (A), endothelial cells (EC) and co-cultured amniocytes and endothelial cells (EC+A). Ratio of phosphorylated (p) VEGFR2 at tyrosine 1175 versus total VEGFR2 were altered in endothelial cell (EC) when co-cultured with patient cells (3.4-fold) compared to endothelial cells co-cultured with control cells (1.3). SMA is an amniocyte marker.