HIRA, a DiGeorge Syndrome Candidate Gene, Is Required for Cardiac Outflow Tract Septation

Michael J. Farrell, Harriett Stadt, Kathleen T. Wallis, Peter Scambler, R. Lester Hixon, Raymond Wolfe, Linda Leatherbury, Margaret L. Kirby

Abstract—DiGeorge syndrome (DGS) is a congenital disease characterized by defects in organs and tissues that depend on contributions by cell populations derived from neural crest for proper development. A number of candidate genes that lie within the q11 region of chromosome 22 commonly deleted in DGS patients have been identified. Orthologues of the DGS candidate gene HIRA are expressed in the neural crest and in neural crest–derived tissues in both chick and mouse embryos. By exposing a portion of the premigratory chick neural crest to phosphorothioate end–protected antisense oligonucleotides, ex ovo, followed by orthotopic backtransplantation to the untreated embryos, we have shown that the functional attenuation of cHIRA in the chick cardiac neural crest results in a significantly increased incidence of persistent truncus arteriosus, a phenotypic change characteristic of DGS, but does not affect the repatterning aortic arch arteries, the ventricular function, or the alignment of the outflow tract. (Circ Res. 1999;84:127-135.)

Key Words: DiGeorge syndrome ■ HIRA ■ persistent truncus arteriosus

On the basis of the organs and tissues affected and evidence from experiments on animal models, the congenital diseases DiGeorge syndrome (DGS) and velocardiofacial syndrome (VCFS) are believed to be caused by deficient contributions from the cranial neural crest.1 The most common distinct features of these syndromes include interrupted aortic arch, outflow tract malformation, hypoplastic thymus, and hypoparathyroidism.2 Significantly, the ablation of cardiac neural crest in chick embryos results in phenotypes that closely resemble those found in DGS patients.3 This implies that studies on chick cardiac neural crest may provide insight into the molecular mechanisms that underlie these diseases. Since >85% of DGS and VCFS patients carry microdeletions in the q11.2 region of chromosome 22, much effort has been devoted to identifying genes that lie in this region.4–12 A number of candidate genes, including HIRA, which encodes a putative chromosome remodeling protein, have been mapped to this region.3,13–15 It has been shown that HIRA orthologues are expressed in the neural crest and in neural crest–derived tissues in both chick and mouse embryos,6,15 making HIRA a particularly strong candidate gene. In the chick, cHIRA is expressed in the developing neural plate, the neural tube, the neural crest, and the mesenchyme of the head and pharyngeal arches.

Both DGS and VCFS are autosomal dominant genetic diseases.16 Cases linked to the q11.2 region of chromosome 22 are apparently caused by a haploinsufficiency of 1 or more genes that lie in this region. Although the deletions found in this region are typically ≈3 Mb, Gong et al17 have identified a DiGeorge critical region that is 250 kb, on the basis of shortest region of deletion overlapping. Complicating matters, a second DiGeorge critical region, distinct from the first, has been identified in a different patient population.18 Both critical regions are at the proximal end of the typical 3-Mb deleted region. Perhaps regulatory elements in 1 region affect gene expression in the other. One patient with DGS in association with a balanced translocation involving 22q11 sequences has been described, but this chromosome rearrangement does not disrupt any protein-encoding gene,9 supporting the view that a long-range regulatory element lies in this region of the genome.19 It seems likely, given the broad scope of the phenotypes found for both DGS and VCFS, that the hemizygosity of multiple genes in these regions is responsible for the variations in phenotypic features observed in these syndromes. Several genes mapped to chromosome 22q11.2 are now the subject of intense investigation.

One way to determine whether a given candidate gene is actually involved in the etiology of a human genetic disease is to examine the phenotypic effect of functional disruption of the orthologous gene in an animal model. One popular model in which to study the function of a disrupted gene is the mouse, in which homologous recombination in embryonic stem cells allows any gene of interest to be targeted for disruption. Unfortunately, because of the pleiotropic effects of many genes and the subtle and complex manner in which
pleiotropy affects development, the phenotypic effect in mice harboring this sort of gene disruption cannot always be related to lack of expression in a particular tissue.

Antisense oligonucleotides designed to hybridize to specific mRNA sequences have been used to attenuate the function of numerous genes both in vitro and in vivo. Recently, antisense oligonucleotides have been used to disrupt the function of developmentally regulated genes in specific tissues of chick embryos. This method can be particularly useful for studies on chick premigratory cardiac neural crest, in which techniques for explanting and backtransplanting tissue are well established. In the chick, neural crest, in which techniques for explanting and backtransplanting tissue are well established. In the chick, gene function can be specifically attenuated in the cardiac neural crest by extirpation of the neural folds from the level of the otic placode to somite 3, placing the folds in a solution containing the antisense oligonucleotide for a short time and then backtransplanting them into the same embryo. Unlike typical mouse null mutants generated by homologous recombination, this technology allows gene function to be studied in a specific tissue, from a defined point in development, and may allow researchers to begin to tease out the genetic pathways involved in cardiac neural crest development.

By exposing the premigratory chick cardiac neural crest to antisense oligonucleotides against cHIRA, the chick HIRA orthologue, ex ovo, followed by orthotopic backtransplantation to untreated hosts, we have shown that the functional attenuation of cHIRA in the cardiac neural crest results in a phenotypic change characteristic of DGS. Specifically, treatment of chick cardiac neural crest with antisense oligonucleotides to cHIRA results in a significant portion of treated embryos developing persistent truncus arteriosus (PTA). This result generally supports the hypothesis that the cardiovascular features of DGS and VCFS are caused by deficient or related to lack of expression in a particular tissue.

Materials and Methods

Operative Strategy and Explant Incubation

Fertilized Arbor Acre chicken eggs were incubated at 37°C and 70% humidity for about 30 hours or until they reached Hamburger-Hamilton stage 10. The eggs were windowed, and the embryos were stained with neutral red. The vitelline membrane was torn over the rhombencephalon, and the alar plate extending from the mid-otic placode to somite 4 was removed as a single piece. If the neural folds separated, the embryo was discarded. The alar plate was incubated for 1.5 hours at 37°C with 5% CO₂ in a minimal volume of guanidinium isothiocyanate and frozen in liquid nitrogen. After thawing, 0.5 mL RNAzol was added to each set of folds and then incubated on ice for 1 hour. One-tenth volume of chloroform was added, and the mix was incubated another 15 minutes on ice followed by centrifugation at 12,000g for 15 minutes. One-half volume of isopropanol and 10 μg of glycogen were added to the aqueous fraction, and the RNA was precipitated. The RNA was washed twice with 1 mL of 85% alcohol and then resuspended in 1 mL of 70% ethanol containing 120 mmol/L sodium acetate. After overnight incubation at −70°C, the RNA was precipitated and washed twice with 1 mL of 70% ethanol.

Approximately 1 μg of total RNA isolated from either antisense or control oligonucleotide–treated explants, as spectrophotometrically determined, was reverse transcribed using random hexamer primers and a Gibco-BRL SuperScript RNase H Reverse Transcriptase kit (catalogue No. 18053-017), according to the manufacturer’s instructions. One μL of the first-strand cDNA produced was used as a template for the PCR reactions. PCRs were performed in 30-μL reactions containing both Hoxb4 and Hira primers, shown below, at a final concentration of 1 μmol/L. A touchdown protocol was followed, initially annealing at 61°C and proceeding in 1°C steps to 56°C followed by 22 cycles at this annealing temperature. This number of cycles produced product in the linear range. Reactions were run on 2.5% NuSieve agarose gels, blotted, and hybridized with radiolabeled Hoxb4 and Hira probes. Filters were exposed to phosphor imaging screens, and signals were quantified using ImageQuant software (Molecular Dynamics). Primers on which PCR was performed were the following: Hira (forward), 5’-TGGATATGGCTCCCTGGTCTG-3’; Hira (reverse), 5’-CAGATGAGGTTGCAAGCCAAG-3’ (taken from chick Hira sequence, GenBank accession No. X99375); Hoxb4 (forward), 5’-CCAGACGCGTGTTATCCTCC-3’; and Hoxb4 (reverse), 5’-CTTCTTCCATTTCATCTCG-3’.

RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For each oligonucleotide, 6 treated neural folds were collected in a minimal volume of guanidinium isothiocyanate and frozen in liquid nitrogen. After thawing, 0.5 mL RNAzol was added to each set of folds and then incubated on ice for 1 hour. One-tenth volume of chloroform was added, and the mix was incubated another 15 minutes on ice followed by centrifugation at 12,000g for 15 minutes. One-half volume of isopropanol and 10 μg of glycogen were added to the aqueous fraction, and the RNA was precipitated. The RNA was washed twice with 1 mL of 85% alcohol and then resuspended in 1 mL of 70% ethanol containing 120 mmol/L sodium acetate. After overnight incubation at −70°C, the RNA was precipitated and washed twice with 1 mL of 70% ethanol.

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Immunochemistry

Neural folds that had been explanted, treated with control or antisense oligonucleotides, and cultured for 24 hours at 37°C in α-MEM containing 5% horse serum were placed in methanol at −20°C overnight. After washing twice in PBS, cells were incubated in 5% goat serum/PBS for 30 minutes followed by a 1-hour incubation in the HIRA antibody (a gift from J.H.C. Meijers, University Hospital, Utrecht, The Netherlands) diluted in PBS at 1:200 or in primary antibody (Sigma) diluted at 1:1000. The fixed neural crest cells were washed in PBS before a 1-hour incubation in FITC-labeled goat anti-mouse antibody (Southern Biotechniques) diluted at 1:50 in PBS. Finally, the neural crest cells were washed once in PBS before Gel Mount (Fisher) was applied to preserve the fluorescence.

Ink Injections

Pelikan India ink was injected into a vitelline vein using a capillary pipette pulled to a fine diameter. The ink was injected using gentle, positive pressure until it filled the intravascular compartment. The embryos were dissected from surrounding membranes and immersion fixed in neutral buffered formalin overnight, dehydrated, and cleared in benzyl benzoate:methylsalicylate (1:1).
Microcinemophotography

Real-time video analysis was used to determine the shortening fractions, ejection fractions, ventricular diastolic and systolic areas, and cardiac outputs as described by Leatherbury et al. for neural crest–ablated chick embryos. The chick embryos were selected for videomicroscopy at stage 18, when the heart is a looped tube with a well-formed outflow tract and bilaterally symmetrical aortic arch arteries 2, 3, and 4. Videophotography was performed using a high-speed, digital video camera (Kodak 100 HRC digital imager) mounted on a stereoscopic microscope (Olympus Corporation of America). The heart cycle was recorded at a frame rate of 100 frames per second, for 10 seconds, resulting in 40 frames per cardiac cycle for a typical embryo. Cardiac measurements were made according to the methods previously described by Leatherbury et al. Cardiac indices were then calculated. These included the shortening fraction, ejection fraction, stroke volume, heart rate, and cardiac output of the cardiac outflow segment. All image analysis was performed independently by 2 investigators, who were blinded to the experimental status of the embryos. Parameters that were significantly different by ANOVA were further tested by Tukey’s honest significant difference procedure. Significant interactions were considered to be \( P < 0.05 \).

Western Blot Hybridizations

Embryos (one 10.5-day-old mouse embryo, 22 stage 14 chick embryo heads, and 10 stage 22 whole embryos) were collected in PBS and then homogenized in an equal volume of lysis buffer (50 mmol/L Tris, pH 7.4, containing 0.25% deoxycholate, 125 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L PMSF, and 1 \( \mu \)g/mL aprotinin). The homogenate was centrifuged at 5°C at 15,800 g for 5 minutes. Protein concentrations were determined on the supernatant using the Bradford protein assay with BSA as a standard. Twenty micrograms of each extract were fractionated on a 12% SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose. The membrane was blocked overnight at 5°C in PBS containing 0.1% Tween 20, 5% BSA, and 0.5% nonfat milk and then incubated with the primary antibody in PBS containing 0.1% Tween 20 and 0.5% BSA. After washing with 0.1% Tween 20, 5% BSA, and 0.05% Tween-20, the membrane was incubated in a goat anti-rabbit secondary antibody with an alkaline phosphate tag for 30 minutes at room temperature. After washing, color was developed using NBT and BCIP.

Results

Phenotypic Change Caused by cHIRA Antisense Treatment of Cardiac Neural Crest

To determine the functional role played by cHIRA in the cardiac neural crest, neural folds at the level of midotic placode to somite 3 were explanted into 10 \( \mu \)L of \( \alpha \)-MEM containing 2 nmol of 1 of 2 phosphorothioate-protected antisense oligonucleotides (as1 and as2) designed to hybridize to the portion of the mRNA that spans the ATG encoding the first amino acid in the cHIRA protein. A phosphorothioate-protected missense oligonucleotide of the same nucleotide composition as as1, but with nearest neighboring nucleotides swapped, (mis2) was used in control experiments.

Examination of day 8 embryos, 7 days after backtransplantation of neural folds treated with the cHIRA antisense oligonucleotides, revealed a significant increase in PTA as compared with embryos in which the neural folds had been treated with a missense oligonucleotide (Figure 1). Eight of 12 embryos in which the neural folds had been treated with as2 and 11 of 17 embryos treated with as1 developed PTA (Figure 1). Only 1 of 8 embryos treated with mis2 developed PTA (Figure 1). In total, 66% of the embryos in which folds had been treated with an antisense oligonucleotide developed PTAs, whereas 12.5% of the embryos in which folds had been treated with a control oligonucleotide and 25% of the embryos treated with medium alone developed PTAs (Figure 1). These results should be considered in light of the fact that only 90% of chicks with complete cardiac neural crest ablation develop PTAs.

While a high incidence of PTAs was found in embryos in which the chick neural crest had been treated with the cHIRA antisense oligonucleotide, the percentage of those embryos in which the common outflow vessel arose completely from the right ventricle was much lower than is typically found after cardiac neural crest ablation. Seven hearts from the antisense group that had developed PTAs were sectioned and examined microscopically to determine outflow tract alignment. Of these hearts, 5 (71%) had normally aligned outflow tracts (Figure 2). The outflow tracts of the other 2 hearts were found to arise entirely from the right ventricle. This result contrasts with that found in a study in chick neural crest–ablated embryos in which \( >80\% \) of outflow tracts originated from the right ventricle. In our study, it appeared that smaller ventricular septal defects (VSDs) located immediately beneath the semilunar valves correlated with normal alignment, while large VSDs were found in hearts in which the outflow vessel arose completely from the right ventricle. The PTAs examined by histology were all type 1, in which a short main pulmonary trunk branched from the truncus and gave rise to 2 pulmonary arteries. The single arterial valve had 5 cusps, which is expected in avian PTA.

To determine whether aortic arch artery formation was affected by the antisense treatments, ink injections were performed on stage 22 embryos (Figure 3). Although some minor variations were observed, no significant differences were observed between embryos in the antisense and missense groups, suggesting that the anomalies were induced by technical procedures that are common to both groups, rather than attributable to the antisense treatment. In addition, aortic arch artery anomalies that were observed at early stages appeared to resolve by day 8, since patterning of the great arteries was normal.

![Figure 1](image-url). The number of embryos with normal hearts or with PTA 8 days after incubation. The cardiac neural crest was treated with the various oligonucleotides or with vehicle alone. The bar graph shows the number of normal hearts and the number of PTAs obtained in each group. as1 and as2 are 2 different cHIRA antisense oligonucleotides; mis2 is a missense oligonucleotide; media indicates vehicle.
Attenuated Expression of cHIRA in Cardiac Neural Crest

To show that the increased occurrence of PTA correlated to a decrease in cHIRA expression, experiments were carried out to determine the effect of antisense treatment on both cHIRA mRNA and protein levels in the cardiac neural crest. Six hours after backtransplantation, when the treated neural crest had reattached to the embryo, the level of cHIRA mRNA expression in the cardiac neural crest was examined. At this stage, cHIRA expression in the cardiac neural crest, as determined by in situ hybridization, was low, making comparison of cHIRA expression in antisense- and missense-treated cardiac neural crest by this method problematic (data not shown). However, quantitative RT-PCR performed on mRNA isolated from neural folds immediately following oligonucleotide treatment showed a ≈26% decrease in cHIRA mRNA levels in antisense-treated folds (Table 1) as determined by autoradiodensitometry (Figure 4). These folds were treated exactly as those that were used in backtransplantations. RNA was isolated from the folds immediately fol-

Figure 2. Chick hearts at 8 days of incubation. a through e, Semiserial sections of a normal heart from the missense-treatment group. f through j, Semiserial sections of a heart from the HIRA antisense-treatment group. Selected sections through the outflow tract of the heart from the antisense group show that the septation defect extends from the region of the aortic sac through the distal conus, which encompasses most of the outflow septum. The subvalvular distal conus (panel g, ✽) remains open and is known to be populated by cardiac neural crest cells normally. h and i, Sections through the single arterial valve, which has 5 leaflets typical of a chick PTA. j, Base of the arterial trunk. The sections from this heart with PTA can be compared with sections through the normal heart that show a robust outflow septum, including an aortic and a pulmonary semilunar valve, each with 3 leaflets. The alignment of the aortic and pulmonary channels in the common outflow vessel is normal, with the posteriorly positioned aortic channel arising from the left ventricle and the anteriorly positioned pulmonary channel arising from the right ventricle. These channels spiral around each other dextroanteriorly (aorta) and sinistrodorsally (pulmonary), giving off the brachiocephalic and right and left pulmonary arteries in a normal pattern. There is complete closure of the interventricular communication and formation of a normal pulmonary infundibulum. The arrow indicates the PTA. P indicates pulmonary trunk; A, aorta; and RV, right ventricle.

Figure 3. Aortic arch artery patterns in ink-injected embryos. Embryos in which the cardiac neural crest had been treated with as2 were injected with India ink at stage 22. This is soon after the caudal arch arteries have opened. The arch artery pattern (a) showed slight variations (arrow) from normal (b) in some embryos, but these anomalies appeared to resolve by incubation day 8, since no variations were found in analysis of these older embryos. Most embryos treated with the antisense oligonucleotide developed normal arch-artery patterns.
lowing the 1.5-hour oligonucleotide treatment in α-MEM. The 26% decrease in cHIRA mRNA in neural folds treated with antisense oligonucleotide, while significant, may not completely reveal the extent to which the antisense oligonucleotide attenuates functional cHIRA in the cardiac neural crest. In addition to effects on mRNA levels, the cHIRA antisense oligonucleotides may significantly reduce protein levels by inhibiting translation of the cHIRA mRNA. To determine the effect of antisense treatment on cHIRA protein expression, neural folds treated with antisense or missense oligonucleotides were maintained in culture medium for 24 hours. At 24 hours, the neural folds were fixed, and the level of cHIRA expression in the neural folds was assayed by immunocytochemistry (Figure 5). The antibody used in these assays was made against the mouse HIRA protein, but Western blot analysis showed that this antibody also recognized a chick protein of the correct size (Figure 5a). A second smaller chick protein recognized by the antibody is likely to be a degradation product of cHIRA. Compared with neural folds treated with the missense oligonucleotide, those neural folds treated with the antisense oligonucleotide showed a marked decrease in the level of cHIRA protein as detected by immunocytochemistry in migrating neural crest cells (Figure 5b through 5e). To ensure that the effect of antisense oligonucleotides on cHIRA protein expression was not non-specific, α–smooth muscle actin expression was analyzed similarly in cultured neural crest cells after oligonucleotide treatment. In culture, α–smooth muscle actin is generally

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*MSX2 served as the control, and all other values are normalized to results found in that group.
†Each neural fold in a group was treated with either 2 nmol (×2) or 4 nmol (×4) of the oligonucleotide.
‡eg, 63/100 × 100/85 = 0.74.

Figure 4. cHIRA RNA expression in cardiac neural crest after oligonucleotide treatment shows the results of autoradiograph densitometry quantification for cHIRA RNA by RT-PCRs performed on neural folds treated with either the as1 or mis2 oligonucleotide. RNA was isolated from cardiac neural crest immediately following the oligonucleotide treatment. RT-PCR for HoxB4 RNA was used as a control for quantification in these experiments. There was a 26% reduction in cHIRA amplification after treatment of neural folds with the as1 oligonucleotide as compared with cHIRA amplification after treatment with the mis2 oligonucleotide. AS×4, MS×2, and AS×2 indicate treatment with 4 mmol/L as1, 2 mmol/L mis2, and 2 mmol/L as1, respectively.
expressed in both smooth muscle cells and neural crest cells. No differences in expression of α-smooth muscle actin were observed between the antisense- and missense-treated cells (data not shown).

Ventricular Function

No significant differences in the hemodynamic variables were observed in embryos with attenuated cHIRA in the cardiac neural crest (Table 2). Although there was a significant difference in the heart rates observed in the 3 groups that underwent backtransplantations of the premigratory neural crest versus the sham-operated control group, this does not support the view that attenuation of cHIRA gene expression affects embryonic hemodynamics, since there was no evidence of decreased contractility caused by attenuation of cHIRA expression, as is seen following neural crest ablation in the chick.

Discussion

HIRA, initially called TUPLE1, was isolated through positional cloning and has been mapped to the DiGeorge critical region. A number of attributes make HIRA a particularly strong DGS candidate gene. HIRA is hemizygous in all but 1 DGS patient with a known 22q11 deletion and maps within the more distal of the 2 published critical regions. The chick and mouse orthologues of HIRA are expressed at relatively high levels in embryonic regions that contain migrating neural crest cells, particularly those emanating from rhombomeres 4 and 6. cHIRA is expressed throughout the rostral mesenchyme, but with particularly high levels of expression in regions that contain neural crest cells. At later stages, expression is strong in neural crest–derived regions of the head and in the pharyngeal arches and pouches. This expression pattern is intriguing, given the fact that the cardiovascular and glandular structures affected in DGS depend on contributions from pharyngeal arches 3 and 4.

The hypothesis that DGS is due to a defective contribution to the affected structures by neural crest–derived cells is supported by several lines of evidence. The neural crest, which originates in the neural folds at the dorsalmost aspect of the neural tube, contributes to the development of numerous neural and nonneural structures affected in DGS patients. Studies on neural crest migration have shown that the rostral neural crest makes significant contributions to the formation of the developing head and pharyngeal arches, in which the neural crest–derived cells differentiate into mesenchymal cell precursors that will contribute to the head and in the pharyngeal arches and pouches. Removal of the neural crest at this level before migration leads to defects in the cardiac neural crest–derived conotruncal defects found in DGS patients. OF particular interest is the fact that all of the heart defects seen after neural crest ablation are also part of the DGS phenotype. PTA, which is the hallmark of cardiac neural crest ablations, is rare in humans with nonsyndromic congenital heart disease.

When the cHIRA gene expression is attenuated in the premigratory chick cardiac neural crest, PTAs are produced in a significant portion of chick embryos. Therefore, HIRA remains a viable candidate gene to underlie the neural crest–related conotruncal defects found in DGS patients. Cardiac neural crest absence during early cardiovascular development results in a wide spectrum of cardiovascular abnormalities. Previous in vivo microcinematography stud-

<table>
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*P<0.001, †P=0.017.

AS indicates antisense; MIS, missense; CO, cardiac output (mL/min); HR, heart rate (bpm); and EF, ejection fraction.
ies have shown that neural crest–ablated embryos have morphological and functional changes long before structural congenital heart defects, such as truncus arteriosus, are apparent.\(^3\) Morphologically, these embryos often exhibit incomplete looping of the cardiac tube, altered conotruncal shape, and dilated ventricles at stage 18.\(^3\) The most striking functional abnormality observed in neural crest–ablated chick embryos is decreased ventricular contractility.\(^3\) Recently, 2 aspects of early myocardial development, excitation–contraction coupling and the assembly of the contractile apparatus, have been shown to be defective in cardiac neural crest–ablated embryos, indicating that early myocardial development is influenced by the cardiac neural crest. This influence of the neural crest on myocardial development is both temporally and spatially isolated from the role of the neural crest in outflow tract septation.

\(c\)HIRA gene attenuation in the cardiac neural crest causes an increased incidence of PTA, a structural defect associated with cardiac neural crest ablation, but it does not affect ventricular function, arch-artery development, or outflow tract alignment. Our results indicate that outflow tract alignment does not depend on septation and is more likely due to defective myocardial development. The absence of ventriculofacial abnormalities or outflow misalignment in these hearts suggests that attenuation of \(c\)HIRA expression in the cardiac neural crest does not affect the role played by neural crest in early myocardial development. Furthermore, the fact that outflow tract alignment tends to be normal, in this case, in the absence of outflow tract septation, supports the hypothesis that early myocardial development plays a major role in outflow tract alignment.

In previous experiments, antisense oligonucleotides to a paralogous \(Hox\) gene family were shown to affect development of the aortic arch arteries significantly but did not cause a high incidence of PTA,\(^2\) showing that arch-artery development and conotruncal septation are not dependent on one another. The current experiments showing that defective outflow septation can occur in the absence of arch-artery anomalies supports this finding. It would be interesting to determine whether outflow tract alignment or ventricular function is affected in embryos with isolated arch-artery anomalies.

The homology of HIRA to proteins known to play roles in regulating chromatin assembly suggests a possible role for HIRA in the regulation of transcription through alterations in chromatin structure. HIRA encodes a protein with no apparent DNA-binding domain, but with a number of WD (Trp-Asp) repeats, helical protein structures forming propeller-like structures and thought to be involved in protein-protein interactions.\(^3\) The strong homology between HIRA and HIR1 and HIR2 suggests that HIRA may also function in the regulation of histone synthesis in phase with the cell cycle.\(^3\) The dynamic \(Hira\) expression pattern and its homology to other genes, such as TUP-1, suggest a possible role in the global regulation of gene transcription via transcription factor/histone interactions.\(^4\) TUP-1 is known to interact with histones and with the homeodomain protein \(\alpha 2\). Recently, HIRA has been shown to interact directly with the histones H3 and H2B,\(^9\) supporting the view that HIRA may act as a regulator of chromatin structure.

Most intriguing is the report of an interaction between HIRA and the transcription factor Pax3.\(^5\) A Pax3 mutation in the Splotch mouse results in a cardiovascular phenotype that closely resembles DGS. Mice that are homozygous for this Pax3 mutation die in utero or neonatally with cardiovascular and neural tube defects. Haploinsufficiency of HIRA in DGS patients, by causing altered stoichiometries in transcription factor complexes, may be crucial to the etiology of the disease.

The cardiovascular phenotype seen in the chicks in which \(c\)HIRA had been functionally attenuated in the cardiac neural crest is similar to that found in the Sox-4–null mutant mouse.\(^5\) Sox-4 is a member of the sex-determining region \(Y\)-related high-mobility group transcription factors, which regulate transcription by altering chromatin structure. Since Sox-4 is expressed in the endocardial ridges rather than neural crest, in this instance, it has been suggested that the defective development of the outflow tract septum is due to a disturbance of inductive processes between the ingrowing neural crest–derived cells and the endocardial ridges. Although Hira and Sox-4 expression do not coincide, each may play a role in any inductive process between the endocardial ridges and the cardiac neural crest. Other structures that depend on contributions from the cardiac neural crest for proper development are unaffected in these mice. Null mutations in 2 other gene families show a wider spectrum of cardiovascular defects, which are probably caused by deficiencies in the cardiac neural crest. Transforming growth factor (TGF-\(\beta\))-\(\beta 2\)-null mice have a similar although milder spectrum of defects, including a large percentage of mutant mice that harbor VSDs.\(^5\) Other aspects of the phenotype, such as craniofacial defects, including cleft palate, support the view that a primary cause of the heart defects found in these mice is deficient or defective contributions by cranial neural crest–derived cell populations to cardiovascular development. TGF-\(\beta\)s are known to affect the migration, differentiation, and cell adhesion of cranial neural crest cells and also to modulate production of certain components of the extracellular matrix as well as to specifically enhance proliferation of neural crest–derived smooth muscle cells. Abnormal development of the aortic arch arteries is not found in either the Sox-4–null mouse or the TGF-\(\beta 2\)–null mouse.\(^5\) Retinoic acid receptor (RAR) \(\alpha/RAR(\beta\) or \(\gamma\) and retinoid X receptor-\(\alpha/RAR(\alpha, \beta, \text{or } \gamma)\) compound mutant mice have a spectrum of phenotypes affecting a variety of structures that depend on neural crest–derived cells for proper development, including conotruncal defects and defective patterning of the great arteries.\(^4\) The conotruncal defects in these mice include PTAs. It will be interesting to determine whether HIRA expression is affected in any of these mutants or, alternatively, whether attenuation of HIRA expression affects expression of any of these genes.

Abnormal remodeling of the aortic arch arteries and right ventricular origin of the common arterial trunk are hallmarks of >90% of embryos with cardiac neural crest ablation in our
experience. 34 Manner et al 35 performed the neural crest extirpation in unstained embryos after dorsal closure of the neural folds and found somewhat different results. The embryos had a high incidence of abnormal remodeling of the aortic arch arteries. Since the hearts were analyzed by scanning electron microscopy, comparisons with the present results are difficult. They typically showed high subpulmonary VSDs, with only 30% of the embryos having PTA. In some of the embryos with high VSDs, a fissure was seen between 2 well-formed semilunar valves, each having 3 leaflets. This same type of fissure was seen in high VSDs produced by unilateral vitelline vein ligation. 56 This fissure, which does not represent PTA because of the presence of 2 well-formed semilunar valves, is not comparable with the PTAs produced after cHIRA treatment of the cardiac neural crest because of the presence of a single valve with 5 leaflets. Using antisense oligonucleotides, we have shown that attenuation of cHIRA expression in chick cardiac neural crest results in a high incidence of PTA, a phenotypic defect characteristic of DGS. These results support the view that attenuated expression of HIRA is involved in the etiology of DGS and that the heart defects associated with DGS can be attributed to contributions from the cardiac neural crest. Our results, in conjunction with the correlation of microdeletions with the broad spectrum of phenotypes seen in these diseases, suggests that more than 1 gene may play a role in the etiologies of DGS and VCFS. Alternatively, deficient HIRA expression in other tissues may be required to generate the other cardiovascular defects observed in DGS patients.

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References

27. Kirby ML, Hunt P, Wallis KT, Thorogood P. Normal development of the cardiac neural crest in mice deficient for HIRA Required for Outflow Tract Septation


HIRA, a DiGeorge Syndrome Candidate Gene, Is Required for Cardiac Outflow Tract Septation

Michael J. Farrell, Harriett Stadt, Kathleen T. Wallis, Peter Scambler, R. Lester Hixon, Raymond Wolfe, Linda Leatherbury and Margaret L. Kirby

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