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A signaling cascade involving endothelin-1, dHAND and Msx1 regulates development of neural-crest-derived branchial arch mesenchyme

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SUMMARY

Numerous human syndromes are the result of abnormal cranial neural crest development. One group of such defects, referred to as CATCH-22 (cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, hypocalcemia, associated with chromosome 22 microdeletion) syndrome, exhibit craniofacial and cardiac defects resulting from abnormal development of the third and fourth neural crest-derived branchial arches and branchial arch arteries. Mice harboring a null mutation of the *endothelin-1* gene (*Edn1*), which is expressed in the epithelial layer of the branchial arches and encodes for the endothelin-1 (ET-1) signaling peptide, have a phenotype similar to CATCH-22 syndrome with aortic arch defects and craniofacial abnormalities. Here we show that the basic helix-loop-helix transcription factor, dHAND, is expressed in the mesenchyme underlying the branchial arch epithelium. Further, *dHAND* and the related gene, *eHAND*, are downregulated in the branchial and aortic arches of *Edn1*-null embryos. In mice homozygous null for the *dHAND* gene, the first and second arches are hypoplastic secondary to programmed cell death and the third and fourth arches fail to form. Molecular analysis revealed that most markers of the neural-crest-derived components of the branchial arch are expressed in *dHAND*-null embryos, suggesting normal migration of neural crest cells. However, expression of the homeobox gene, *Msx1*, was undetectable in the mesenchyme of *dHAND*-null branchial arches but unaffected in the limb bud, consistent with the separable regulatory elements of *Msx1* previously described. Together, these data suggest a model in which epithelial secretion of ET-1 stimulates mesenchymal expression of dHAND, which regulates *Msx1* expression in the growing, distal branchial arch. Complete disruption of this molecular pathway results in growth failure of the branchial arches from apoptosis, while partial disruption leads to defects of branchial arch derivatives, similar to those seen in CATCH-22 syndrome.

Key words: dHAND, Endothelin, Msx1, Branchial arch, Neural crest, Mouse, Human, CATCH-22, Apoptosis

INTRODUCTION

Neural crest cells arise from the neural folds of the developing embryo and migrate to populate diverse structures, including the branchial arches, the sympathetic nervous system and the aortic arch arteries, which traverse through the branchial arches (Bronner-Fraser, 1995). They retain a degree of pluripotentiality during their migration and can eventually differentiate into a variety of cell types, such as bone, cartilage, pigment cells, vascular smooth muscle, neurons and connective tissue (Le Douarin et al., 1994). An event known as ecto-mesenchymal transformation is central to the differentiation of many neural crest cells into mesenchyme-derived tissues (Noden, 1991). How the migratory pattern, condensation, differentiation and proliferation of neural crest cells is controlled at the molecular level has been the subject of intense research (Anderson, 1997).

Development of the cranial neural crest has been studied extensively from an embryological standpoint, in part because of the relatively large number of human congenital syndromes attributed to cranial neural crest defects. For example, Waardenburg's syndrome (Waardenburg, 1951), Treacher Collins syndrome (Sulik et al., 1987) and Pierre-Robin sequence (Dennison, 1965) are characterized by abnormal facial features and are thought to be defects of the first and second branchial arches. Velo-cardio-facial syndrome (Shprintzen, 1978), conotruncal anomaly face (Burn et al., 1993) and DiGeorge syndrome (Van Mierop and Kutsche, 1986) are three conditions that have overlapping phenotypes of facial and cardiac defects. Over 80% of individuals with these three syndromes have microdeletions of one allele of chromosome 22q11.2 (Driscoll et al., 1993). Because they have overlapping phenotypes and likely have a common etiology, it has been proposed that these syndromes be grouped together

and referred to as CATCH-22 (cardiac defects, abnormal facies, thymic hypoplasia, cleft palate, hypocalcemia, associated with chromosome 22 microdeletion) syndrome (Wilson et al., 1993). Based on the observed pattern of defects, CATCH-22 is believed to be a neural crest defect resulting from abnormal development of the 3rd and 4th branchial arches and pouches and their corresponding aortic arch arteries (Lammer and Opitz, 1986). The gene (s) responsible for the defect remains unknown, but the minimal critical region of chromosome 22q11 has been narrowed to 250 kb and several candidate genes identified by sequencing (Li et al., 1994; Gong et al., 1996).

There are six bilaterally symmetric branchial arches, each of which give rise to unique structures in the head and neck (Sadler, 1995). In murine development, the first branchial arch is visible around embryonic day 9.0 (E9.0) just as migrating neural crest cells begin condensing within the arch. The second arch is well formed by E9.5, while the third, fourth and sixth branchial arches become apparent by E10.0. The fifth arch involutes, as does its artery. Neural crest cells give rise to the mesenchyme of the arches, which later differentiates into specific organs and structures of the head and neck. The portion of the cranial neural crest extending from the level of the midotic placode to the third somite, referred to as the cardiac neural crest, populates the aortic arch arteries and is essential for proper remodeling of the arch arteries which results in formation of the mature aortic arch and proximal pulmonary arteries (Kirby and Waldo, 1990). Cell fate studies demonstrate that specific arch arteries contribute to unique segments of the aorta (reviewed in Olson and Srivastava, 1996). Disruption of cardiac neural crest cells in chick embryos resulted in persistent truncus arteriosus (failure of aortopulmonary septation) and interruption of the aortic arch, conditions commonly seen in CATCH-22 (Kirby and Waldo, 1995).

The endothelin family of signaling peptides has been implicated in development of the neural crest (Kurihara et al., 1994). Mice with a null mutation of *endothelin-1*(*Edn1*), the gene encoding endothelin-1 (ET-1), demonstrate craniofacial and cardiovascular abnormalities similar to those associated with CATCH-22 syndrome (Kurihara et al., 1995). ET-1 is a 21 amino acid peptide which has various biological activities including vasoconstriction and cell proliferation (Masaki, 1995). Three isopeptides (ET-1, ET-2 and ET-3) encoded by different loci constitute a gene family and act on two distinct G protein-coupled receptors (ET_A and ET_B) with different affinities (Levin, 1995). Mutant mice lacking ET-3 (Baynash et al., 1994) or ET_B receptor (Hosada et al., 1994) show developmental abnormalities in other neural crest derivatives including melanocytes and enteric neurons, suggesting that two distinct ET signaling pathways contribute to the development of different neural crest lineages. Targeted mutation of an endothelin converting enzyme (ECE-1) results in a phenotype encompassing features of ET_A and ET_B-null mice, suggesting that ECE-1 converts both ET-1 and ET-3 to their active forms (Yanagisawa et al., 1998). *Edn1* is expressed mainly in the epithelium of the pharyngeal arches and the endothelium of the aortic arch artery and cardiac outflow tract (Kurihara et al., 1995). How ET-1 signaling functions in neural crest development remains unclear.

Several transcription factors have also been implicated in controlling development of the neural crest during branchial and aortic arch formation in mice. The clustered *Hox* genes are expressed in an anterior-posterior fashion along the embryo and are expressed in a similar fashion in the branchial arches (Krumlauf, 1993). Mutations in *Pax3* affect the migration of cranial neural crest cells and result in cardiac neural crest defects and craniofacial defects in mice (Epstein et al., 1993; Conway et al., 1997). Mice harboring mutations of the homeobox genes, *MHox* (Martin et al., 1995) and *goosecoid* (Rivera-Perez et al., 1995), among others, also have defects of specific derivatives of the branchial arches.

Members of the basic helix-loop-helix (bHLH) family of transcription factors regulate determination and differentiation of numerous cell types, including skeletal myocytes (Olson and Klein, 1994), neuronal cells (Jan and Jan, 1993; Lee et al., 1995) and hematopoietic cells (Shivdasani et al., 1995). Recently, we found that two novel bHLH proteins, dHAND (Srivastava et al., 1995) and eHAND (Cserjesi et al., 1995), also known as Hed/Thing2 and Hxt/Thing1 (Cross et al., 1995; Hollenberg et al., 1995), respectively, are co-expressed in the developing branchial arches and the aortic arch arteries, as well as the cardiac mesoderm. Deletion of the *dHAND* gene in mice resulted in embryonic death at E11.0 secondary to cardiac failure (Srivastava et al., 1997; Srivastava and Olson, 1997). The right (pulmonary) ventricle was hypoplastic and the aortic arch arteries failed to remain open. Because of *dHAND* and *Edn1*'s expression in the branchial arches and the severe anomaly of the neural-crest-derived aortic arch arteries in *dHAND*-null embryos, we have now performed a detailed analysis of branchial arch development in *dHAND* and *Edn1* mutants. Here we show that *dHAND* expression is limited to the mesenchyme of the distal branchial arches, just below the *Edn1*-expressing epithelium and that, in *Edn1*-null embryos, branchial arch and aortic arch expression of *dHAND* and *eHAND* is markedly downregulated. In the complete *dHAND*-null state, the branchial arches are hypoplastic as early as E9.5. Molecular analyses of *dHAND* and *Edn1*-null embryos demonstrate appropriate migration and differentiation of the neural-crest-derived arch mesenchyme. However, in the absence of dHAND, expression of the homeobox gene, *Msx1*, which has previously been implicated in growth of the branchial arches, is undetectable. Finally, we show that the hypoplasia of the branchial arches in *dHAND*-null embryos is secondary to programmed cell death of the branchial arch mesenchyme. We propose a model in which development of the neural-crest-derived branchial arch ectomesenchyme is mediated by a sequential pathway of epithelial secretion of ET-1, which enhances *HAND* gene expression, which in turn stimulates *Msx1* expression.

MATERIALS AND METHODS

Breeding of mice and genotyping of embryos

Mice heterozygous for *dHAND* or *Edn1* mutations were generated as previously described (Srivastava et al., 1997; Kurihara et al., 1994). Intercrosses of *dHAND* heterozygous mice in the 129SVEV/C57BL6 background were performed. Mothers were killed and their uteri dissected to isolate E9.5 embryos. At this time point, *dHAND*-null embryos were not growth retarded and did not show evidence of cardiac failure. Mice heterozygous for the *Edn1* mutation were similarly bred to obtain E9.5 and E10.5 homozygous null embryos. *Edn1*-null mice were in the 129SV/C57BL6 background. Isolation of

yolk sac DNA from embryos and genotyping of *dHAND* or *Edn1* mutants by Southern analysis was performed as previously described (Srivastava, 1997; Kurihara et al., 1994). Homozygous null and wild-type littermate embryos were isolated and all membranes including the pericardium removed. Embryos were fixed in 4% paraformaldehyde overnight at 4°C and stored in 70% ethanol at –20°C.

In situ hybridization

Whole-mount in situ hybridizations were performed using digoxigenin-labeled antisense riboprobes synthesized from the following cDNAs: dHAND, eHAND, Msx1, Msx2, Dlx2 and MHox. The dHAND cDNA was linearized with *Eco*RI and Sp6 RNA polymerase was used to synthesize the riboprobe. The eHAND cDNA was linearized with *Not*I and T7 RNA polymerase was used for riboprobe synthesis. In situ hybridizations were performed as previously described (Srivastava et al., 1995). Briefly, embryos were prehybridized in hybridization buffer without probe at 60°C for 3 hours; digoxigenin-labeled riboprobes were added and incubated at 60°C for 18 hours. After a series of washes, embryos were incubated with alkaline-phosphatase-conjugated anti-digoxigenin antibodies at room temperature for 1 hour. Following another series of washes, embryos were incubated in a substrate color reaction mixture (Boehringer #1442074) for 12 hours in darkness. Color reaction was terminated by fixing embryos in 4% paraformaldehyde, 0.1% glutaraldehyde.

Histology

Wild-type, *dHAND* or *Edn1*-null embryos hybridized to the various riboprobes were embedded in paraffin after fixation. Transverse sections were made at 5 μm intervals throughout the embryo. Paraffin was cleared in xylene and photographs of sections taken without counterstaining to illustrate color reaction. In some cases, sections were then counterstained with hematoxylin and eosin and photographed.

Bromodeoxyuridine (BrdU) labeling of embryos

BrdU labeling of cells in the S phase of the cell cycle was performed according to the protocol described by Hayashi et al. (1988). BrdU (500 μg/gram of body weight) was injected intraperitoneally into females pregnant (E9.0-E10.0) after *dHAND* heterozygote intercrossing. The females were killed 2 hours after injection, uteri removed and decidual swellings fixed in 4% paraformaldehyde at 4°C overnight and processed for immunohistochemistry. Transverse sections through branchial arches of wild-type and *dHAND*-null embryos were incubated with an anti-BrdU monoclonal antibody (Boehringer Mannheim) at a 1:50 dilution. A rhodamine-conjugated goat anti-mouse IgG (Jackson Labs) antibody (1:25 dilution) was used to visualize anti-BrdU antibodies by confocal microscopy.

TUNEL assay for apoptosis

To visualize apoptotic nuclei in branchial arches in situ, transverse sections of wild-type and *dHAND*-null branchial arches were subjected to terminal transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay (Gavrieli et al., 1992). Sections were incubated for 1 hour at 37°C in 140 mmol/L sodium cacodylate, 1 mmol/litre cobalt chloride, 30 mmol/L Tris-HCl, pH 7.2, 50 U terminal deoxynucleotide transferase and 1 nmol biotinylated dUTP (Boehringer-Mannheim). FITC-conjugated anti-biotin monoclonal antibodies were used to detect biotin-dUTP incorporation in nuclei by confocal microscopy.

RESULTS

Expression of *dHAND*, *eHAND* and *Edn1* in the branchial arches

The cardiac mesodermal expression pattern of *dHAND* and *eHAND* has been well documented (Srivastava et al., 1995, 1997; Biben and Harvey, 1997). Although we have previously reported expression of the *HAND* genes in the branchial arches, here we provide a more detailed analysis of the extent and role of *HAND* expression in the branchial arches. *dHAND* and *eHAND* expression was first detectable at E9.0 in the first and second branchial arches and continued in a similar pattern in each of the branchial arches as they formed between E 9.0 and E10.5 (Fig. 1A,B). However, expression was limited to the distal portion of the arches, with *dHAND* expression being slightly broader than *eHAND*. Histologic analysis revealed that there was no expression of *dHAND* or *eHAND* in the epithelial layer of the branchial arch. Rather, transcripts were concentrated in the neural-crest-derived mesenchyme just underlying the most exterior portion of the arches subjacent to the epithelial layer (Fig. 1C); no expression was detectable in the migratory neural crest cells. The distal region is the leading edge of the growing branchial arch and also expresses the homeobox genes, *Msx1* (Davidson, 1995) and *MHox* (Martin et al., 1995), among others. The localized expression of the *HAND* genes is consistent with the notion that signals emanating from the epithelial layer might induce molecular cascades within the underlying mesenchyme leading to appropriate differentiation. What signals induce *HAND* gene expression in the neural-crest-derived cells just as they condense in the branchial and aortic arches has been unknown; however, *Edn1* is expressed in the epithelial layer of the branchial arches and in the endothelial layer of the the aortic

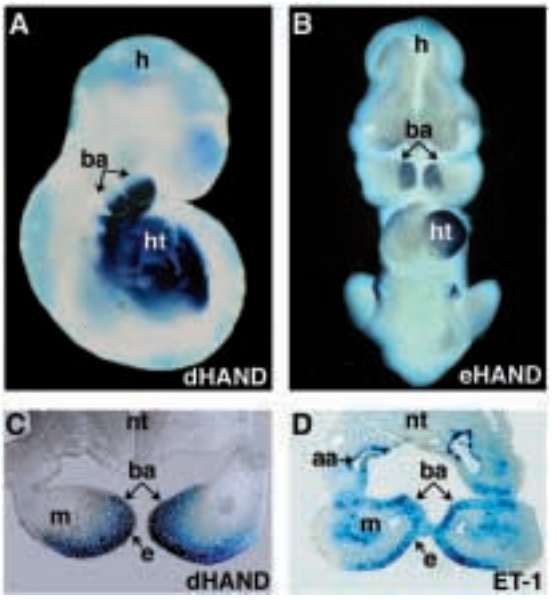

Fig. 1. Branchial arch expression of *dHAND*, *eHAND* and *Edn1*. *dHAND* (A) and *eHAND* (B) are expressed in the distal portions of the branchial arches (ba) as seen in lateral (A) and frontal (B) views, respectively, of E10.0 embryos. *eHAND* expression is more restricted to the medial portion of the arch than *dHAND*. Histological analysis (transverse section) revealed *dHAND* expression in the distal branchial arch mesenchyme (m) subjacent to the epithelium (C), but not in the epithelium (e). In contrast, *Edn1* is expressed specifically in the epithelium of the branchial arches and endothelium of the aortic arch arteries (aa), but not in the branchial arch mesenchyme. h, head; ht, heart; nt, neural tube.

arches (Kurihara et al., 1995; Fig. 1D), adjacent to *HAND* gene expression.

HAND gene expression is downregulated in *Edn1*-null embryos

To determine whether dHAND and eHAND are downstream of the ET-1 signal that arises from the epithelial layer of the branchial arch and endothelial layer of the arch arteries, we examined the expression of *dHAND* and *eHAND* in *Edn1*-null embryos. In E9.5 *Edn-1* mutant embryos, *dHAND* expression in the branchial arches was severely diminished in comparison to that of wild-type littermates (Fig. 2B,F). At E10.0, *dHAND* expression normally observed in the medial portion of the first branchial arch was only detected in a small region close to the midline in *Edn1*-null embryos (Fig. 2C,G). *dHAND* expression in the arch arteries of *Edn1*-null embryos was also lower than that of wild-type embryos (Fig. 2). In contrast, expression in other regions, such as the heart and limb bud, was not different between wild-type and *Edn1* mutant embryos, suggesting that the suppression of *dHAND* expression was specific for cranial neural crest derivatives.

The expression of *eHAND* was similarly affected in *Edn1* mutant embryos. At E10.0, *eHAND* expression in the first and second branchial arches was much lower and restricted to a small region in *Edn1*-null embryos in comparison to wild-type, whereas *eHAND* expression in the left ventricle was unchanged (Fig. 2D,H). This result indicates that *eHAND* expression is also affected by an *Edn1*-null mutation, specifically in neural crest derivatives.

The localization of *HAND* gene expression in the ectomesenchymal cells of the distal, medial branchial arch is complementary to the expression of *Edn1* in the epithelial cells of the branchial arches and the endothelial cells of the aortic arch arteries (Kurihara et al., 1995). It is noteworthy that *Edn1* expression in the branchial arch epithelium is also restricted to the medial half of the arch, corresponding to the expression of *dHAND* and *eHAND*.

If neural crest cells failed to migrate appropriately to the branchial arches, the reduction of *dHAND* and *eHAND* expression in *Edn1*-null mice could be secondary to the absence or reduction in neural-crest-derived cells in the arches. To address this possibility, we examined the expression of *Msx1* and *Msx2*, homeobox genes that are expressed in cranial neural-crest-derived ectomesenchymal cells (Davidson, 1995). The normal pattern of *Msx1* and *Msx2* expression in the branchial arches is similar to that of the *HAND* genes, with a predominance of expression in the distal arch. Unlike the *HAND* genes, *Msx1* and *Msx2* (Fig. 2A,B; unpublished observations) were expressed in normal fashion in *Edn1*-null embryos, indicating that neural crest cells did migrate and were able to express ectomesenchymal markers. Thus, the neural-crest-derived branchial arches cannot fully express *dHAND* and *eHAND* in the absence of ET-1-induced signals and the reduction of their expression is a specific phenomenon.

dHAND-null embryos have hypoplastic branchial arches

Unlike *Edn1*-null embryos, which have a decreased but detectable expression of the *HAND* genes, our disruption of the *dHAND* gene resulted in a complete dHAND-null state. Although *dHAND*-null embryos develop only approximately 20-24 somites before dying of cardiac failure, they do not become growth-retarded until after E9.5. At this time, the embryos are comparable in size and development to wild-type embryos. However, development of the branchial arches is retarded. Unlike wild-type embryos, which have good development of the first and second branchial arches and have begun to form the third and fourth arches by E9.5, *dHAND*-null embryos form a hypoplastic first and second branchial arch and upon histologic examination, have only raised outlines of the third and fourth branchial arches (Figs 3, 4, 6). The growth failure of these neural-crest-derived structures, which normally express *dHAND*, is disproportionate to the development of the rest of the embryo, suggesting that this is a primary phenotype related to the absence of dHAND in the branchial arch. By E10.0, gross and histologic analyses revealed a lack of cellularity in the ectomesenchyme of the first branchial arch and a severely underdeveloped second branchial arch (Figs 3, 4). This phenotype is consistent with the *dHAND*-null effects on the heart (Srivastava et al., 1997),

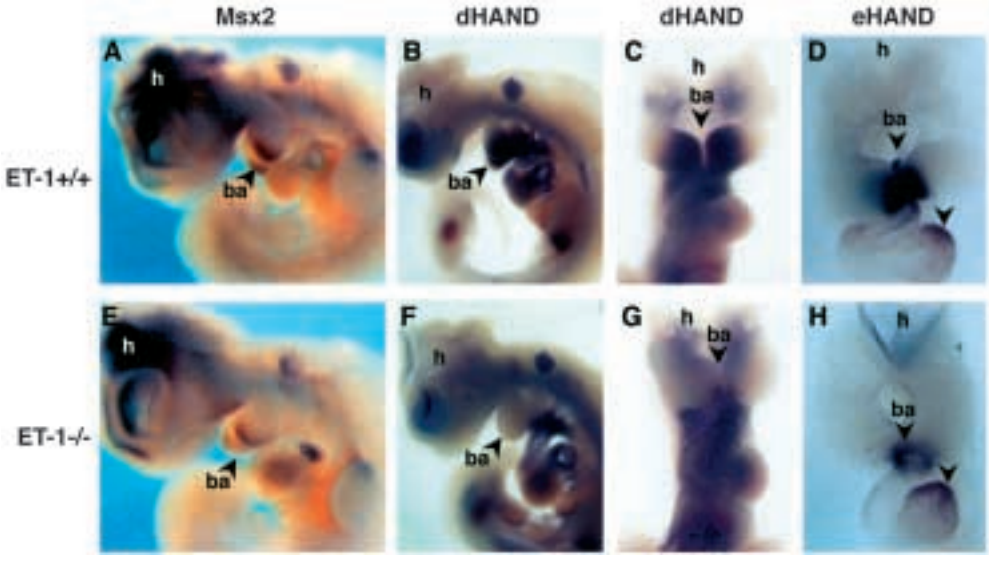

Fig. 2. Downregulation of *HAND* genes in *endothelin-1* (*ET-1*) null branchial arches. Whole-mount in situ hybridization of E9.5-10.0 wild-type (A-D) and *ET-1*-null (E-H) mouse embryos revealed normal expression of *Msx2* (A,E), but downregulation of *dHAND* (B,C,F,G) and *eHAND* (D,H) in the branchial arches (ba). Expression of *dHAND* and *eHAND* is not completely abolished in the medial branchial arches (G,H). *dHAND* and *eHAND* expression in the cardiac mesoderm was unaffected in *ET-1*-null embryos (arrowheads, D,H) as was aortic sac expression. A,B,E,F show lateral views; the head and tail of embryos in C,D,G,H were removed and frontal views shown. h, head.

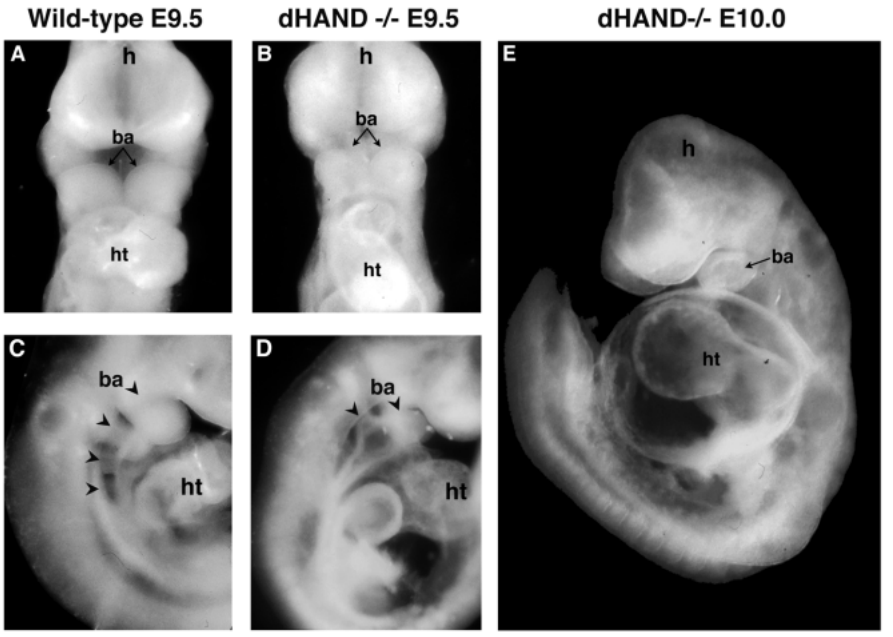

Fig. 3. Branchial arch defects in *dHAND*-null embryos. Frontal (A,B) and lateral (C,D) views of E9.5 wild-type (A,C) and *dHAND*-null (B,D) embryos demonstrate a formed first branchial arch (ba), but hypoplastic second (arrowhead) and undetectable third arch in the mutant. The third and fourth arches are visible in the wild-type and indicated by arrowheads. In a lateral view of an E10.0 *dHAND*-null embryo (E), no growth of the second, third, or fourth branchial arch is visible, while the first branchial arch is becoming hollow from within (see also Fig. 8C). h, head; ht, heart.

where the right ventricle is hypoplastic as well, suggesting growth failure of specific *dHAND*-expressing tissues in the absence of dHAND.

dHAND regulates branchial arch expression of *Msx1*

In order to understand how dHAND might regulate growth of the branchial arches at the molecular level, we examined the expression of several markers of the neural-crest-derived ectomesenchyme in branchial arches of *dHAND*-null embryos (Fig. 5). *MHox* (Martin et al., 1995) which is normally co-expressed in the distal arch with *dHAND* (Figs 5, 6), was expressed at normal levels in E9.5*dHAND* mutant embryos, suggesting that even in the complete absence of dHAND, neural crest cells were able to populate the branchial arch mesenchyme. Similarly, the homeobox genes, *Dlx2* (Qiu et al., 1995) and *Msx2* (Winograd et al., 1997), both required for normal development of branchial arch derivatives later in development, were expressed at comparable levels in wild-type and E9.5*dHAND* mutant embryos (Fig. 5). *Dlx2* maintained its broader expression in the proximal and distal branchial arches (Fig. 6), partially overlapping *Msx* expression at the histologic level. *eHAND* was also expressed at normal levels in the distal branchial arch of *dHAND* mutants as was *Edn1* in the epithelium (data not shown). These results indicate that the migration of neural crest cells into the branchial arch and their initial development is unaffected in the absence of dHAND.

Msx1, another homeobox gene, is normally expressed in the distal branchial arch and limb bud below the epithelial layer (Brown et al., 1993), similar to *dHAND*. In vivo and in vitro studies have implicated Msx1 in controlling growth and development of the branchial arches by mediating epithelial-mesenchymal interactions (Satokata and Maas, 1994; Wang and Sassoon, 1995). Unlike the unaltered expression of *Msx1* in *Edn1*-null mice, which have only a downregulation of dHAND, in the complete absence of dHAND, no expression of *Msx1* was detectable in the branchial arches (Fig. 7). This was in contrast to the normal expression of *Msx1* in the limb

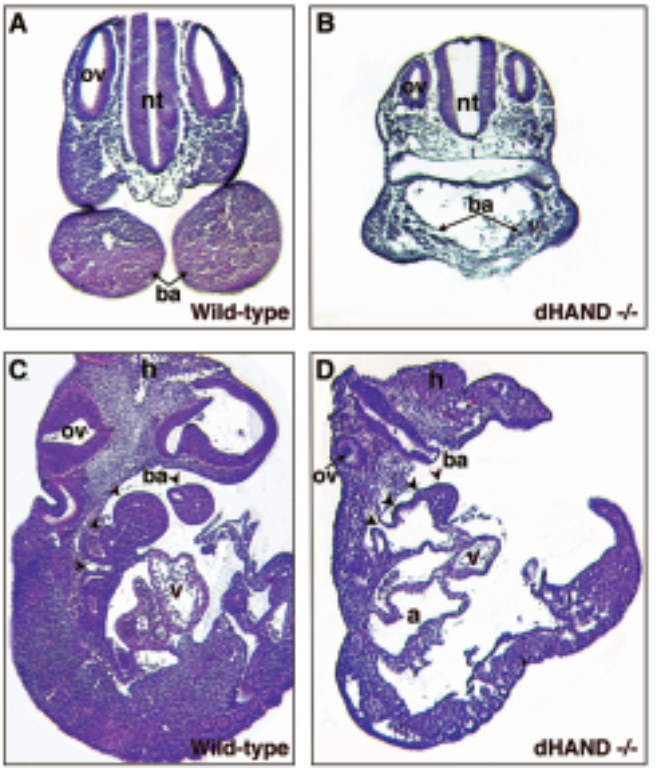

Fig. 4. Histologic analysis of *dHAND*-null branchial arches. Transverse (A,B) and lateral (C,D) sections of wild-type and *dHAND*-null E10.0 embryos were stained with hematoxylin and eosin. Transverse sections at the level of the otic vesicle (ov) revealed hypoplasia of the branchial arch (ba) (B) in *dHAND*-null embryos. Sagittal section through a wild-type embryo revealed four branchial arches (C, arrowheads). In contrast, a comparable section of a *dHAND*-null embryo showed a first branchial arch and only ridges of the second and third branchial arch. The fourth branchial arch artery appears patent in this mutant. v, ventricle; a, atrium; h, head; nt, neural tube.

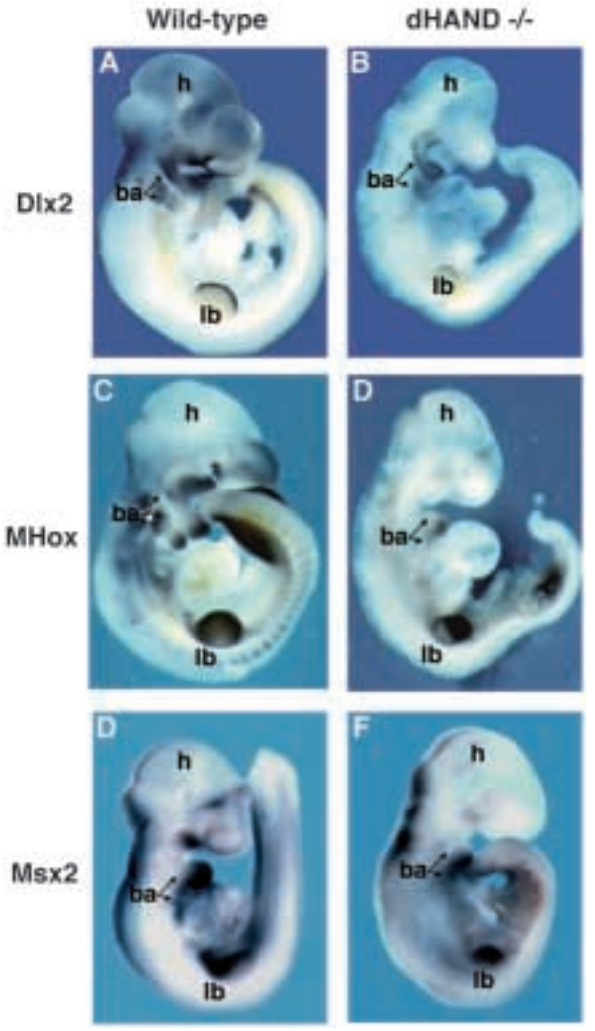

Fig. 5. Expression of branchial arch markers in *dHAND*-null embryos. Whole-mount in situ hybridization revealed normal expression of Dlx2 (A,B), MHox (C,D) and Msx2 (E,F) in the branchial arches (ba) and limb buds (lb) of E9.5 *dHAND*-null embryos (B,D,F) as compared to wild-type (A,C,E). Embryos are shown in lateral views. h, head.

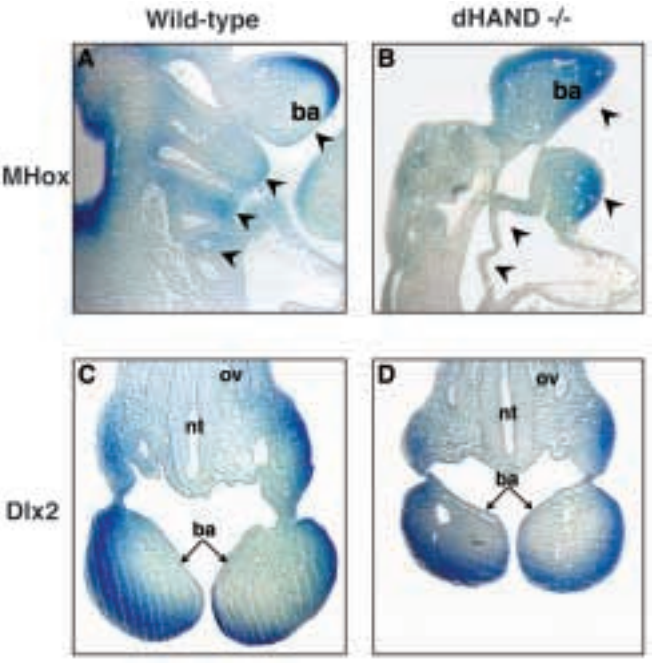

Fig. 6. Histologic analysis of branchial arch markers in *dHAND* mutants. Sagittal or transverse sections were performed on E9.5 embryos after whole-mount in situ hybridization with MHox or Dlx2 riboprobes, respectively. *MHox* expression in wild-type (A) and mutant (B) embryos was comparable and was restricted to the distal mesenchyme subjacent to the epithelium of the branchial arches (ba). Arrowheads indicate additional branchial arches in the wild-type and rudimentary outlines of arches in the mutant. *Dlx2* was also expressed normally in *dHAND*-null embryos and maintained its expression in the proximal and distal mesenchyme. ov, otic vesicle; nt, neural tube.

bud of *dHAND*-null embryos, confirming that the downregulation of *Msx1* was a specific effect of dHAND rather than a non-specific degradation of RNA in the embryo. It is interesting that, although *dHAND* is expressed in the limb bud, *Msx1*, *Msx-2*, *MHox* and *Dlx2* are expressed normally in the early limb bud of *dHAND* mutant embryos (Figs 5, 7). Thus, *Msx1* lies downstream of dHAND in a molecular hierarchy controlling branchial arch growth, but is regulated in a dHAND-independent fashion in the limb bud. Together, dHAND and Msx1 play a role in the earliest stages of development of the distal ectomesenchyme of the branchial arches.

Programmed cell death in *dHAND*-null branchial arches

The mechanism of hypoplasia of the branchial arches in *dHAND*-null embryos could be secondary to a proliferative defect, a differentiation defect and/or programmed cell death (apoptosis). Because the branchial arches expressed other markers of differentiation, we performed BrdU incorporation and TUNEL assays on *dHAND*-null branchial arches to evaluate proliferation and apoptosis, respectively (see Methods). BrdU incorporation was unchanged in *dHAND*-null embryos compared to wild-type (data not shown), suggesting that proliferation was not altered in the absence of dHAND. However, confocal microscopy of sections of branchial arches after TUNEL revealed extensive apoptosis of the first and second branchial arches at E9.5 in *dHAND*-null embryos compared to wild type (Fig. 8A,B). Sections of E10.0 embryos revealed absence of cellularity in the core of the branchial arches where it appeared cell death had already occurred (Fig. 8C). Embryonic regions outside the dHAND expression domain did not exhibit increased apoptosis in the absence of dHAND. These data indicate that dHAND may function in a pathway important for cell survival, although the precise downstream mechanism remains to be determined.

DISCUSSION

Utilizing mice bearing targeted mutations in the genes encoding the signaling peptide, ET-1, and the bHLH transcription factor, dHAND, we have begun to uncover a

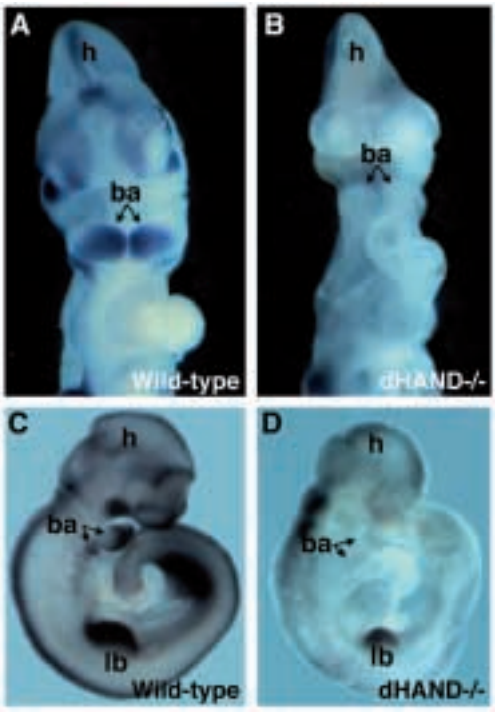

Fig. 7. *Msx1* expression in *dHAND*-null embryos. Whole-mount in situ hybridization revealed complete downregulation of *Msx1* in the branchial arches (ba) of E9.5*dHAND*-null embryos (B,D) compared to wild-type (A,C). Expression of *Msx1* in the limb bud (lb) was unaffected in the mutant (D). Frontal (A,B) and lateral (C,D) views are shown. h, head.

sequential molecular pathway that is critical to the development of derivatives of the cranial neural crest. In the absence of ET-1, *dHAND* and *eHAND* expression is downregulated in cranial neural crest derivatives. This may be responsible, along with other affected factors, for the observed craniofacial and aortic arch defects in *Edn1*-null mice. In the complete *dHAND*-null state, the branchial arches become hypoplastic, apparently secondary to programmed cell death of the mesenchyme. As mesenchymal cells begin to enter the apoptotic pathway, they continue to express markers of differentiation and continue to proliferate, suggesting that activation of cell death in the absence of dHAND may be a primary event. Interestingly, *dHAND*-null branchial arches fail to express the homeobox gene, *Msx1*. *Msx1* is normally expressed at the leading edge of the branchial arch mesenchyme and has been implicated in regulating growth and differentiation of the arches. Together, this pathway appears to regulate development, but not migration, of neural crest cells that are fated to undergo an ectomesenchymal transformation and contribute to the branchial arches and their subsequent derivatives in the head and neck, as well as the aortic arch arteries, which are remodeled to form the mature aortic arch.

How might this pathway regulate development of the neural-crest-derived ectomesenchyme? It is believed that interactions between ectomesenchyme and the surface ectoderm results in differentiation of the mesenchyme into diverse tissues, including cartilage (Ferguson, 1994) and dentine of the teeth (Vastardis et al., 1996; Satokato and Maas, 1994). ET-1 is secreted from the epithelial layer of the branchial arch and the endothelial layer of the aortic arch arteries. Cells in the mesenchyme underlying the epithelium and surrounding the endothelial layer express a G protein-coupled ET-1 receptor, ET_A (Clouthier et al., 1998). Intracellular signaling initiated by the activated ET_A receptor likely contributes to ectomesenchyme differentiation, although the mediators of this process have not been clearly delineated. The *HAND* genes may be mediators of ET-1 signaling, establishing an important link between cell signaling control and transcriptional control of the developing branchial arch. The residual *HAND* gene expression in ET-1 null branchial arches may be secondary to activation by other ETs in which case a knockout of the ET_A receptor may result in complete loss of *HAND* expression. Alternatively, there may be an ET-independent pathway for activation of *HAND* expression, which would later be enhanced by ET signaling. The observation that *dHAND* is regulated independently in neural-crest-derivatives and cardiac mesoderm is consistent with our recent findings which have revealed the existence of separate and independent enhancers that control expression of *dHAND* in these lineages (T. Firulli, J. Charité, D. McFadden, D. S. and E. O., unpublished obervations).

The limited expression of *dHAND* in the distal-most portion of the branchial arches and the downregulation of *Msx1* in the same region of *dHAND*-null embryos indicates that dHAND and Msx1 may be functioning in a common pathway in the distal arch. That *Msx1* is expressed in *Edn1*-null embryos suggests that low levels of dHAND are sufficient to activate Msx1 expression. Two separable enhancers of the *Msx1* gene have recently been described, one controlling first branchial arch expression, while the other controls broader expression of *Msx1*, including expression in the developing limb bud (MacKenzie et al., 1997). The downregulation of *Msx1* in the branchial arch but not limb bud of *dHAND*-null embryos is consistent with the regionally distinct regulatory elements. Whether Msx1 is a direct or indirect target gene of dHAND

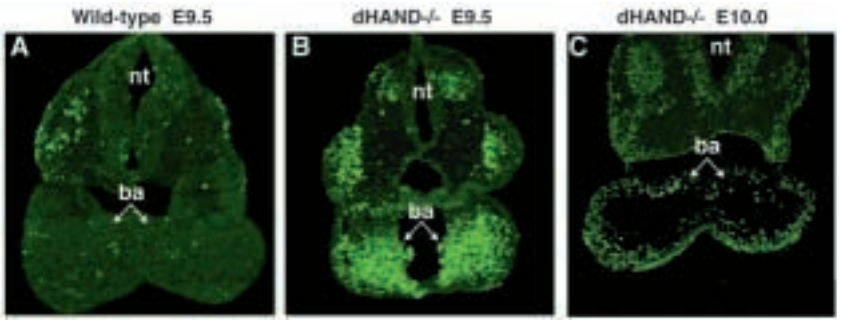

Fig. 8. TUNEL assay of dHAND-null branchial arches. Transverse sections of E9.5 wild-type (A) and *dHAND*-null (B) embryos at the level of the first and second branchial arches (ba) subjected to TUNEL assay revealed extensive apoptosis in the mesenchyme of the branchial arches (fluorescent green). By E10.0, most mesenchymal cells had died and an absence of cellularity in the core of the branchial arch was apparent (C). Images were taken by confocal microscopy. nt, neural tube.

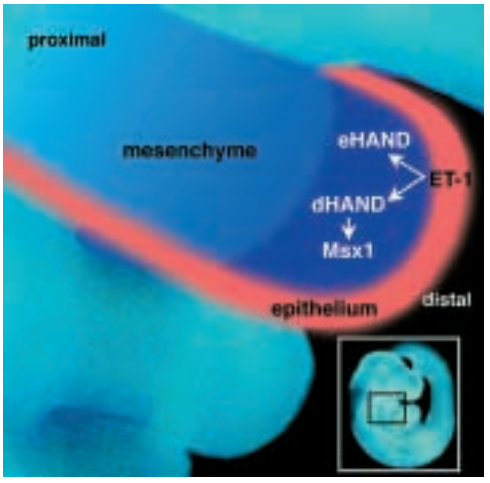

Fig. 9. Model of endothelin-HAND-Msx1 pathway controlling branchial arch growth. Endothelin-1 (ET-1) is secreted from the branchial arch epithelium into the mesenchyme and enhances *dHAND* and *eHAND* expression in the distal mesenchyme. dHAND in turn regulates expression of *Msx1* also in the distal branchial arch. An appropriate balance of Msx1, Msx2 and Dlx2 in the distal arch may be critical for normal growth and development of branchial arch proliferation, differentiation and cell death (apoptosis). Expanded area of interest is shown in insert.

remains to be determined, but it is of note that the 240 bp enhancer for the first branchial arch contains a bHLH recognition site known as an E-box (MacKenzie et al., 1997).

Families of bHLH proteins often function in regulatory cascades where they regulate one another and have overlapping functions in areas of co-expression (Olson and Klein, 1994). *eHAND* expression is unaffected in the *dHAND* mutant, suggesting that eHAND is unable to fully compensate for dHAND in the branchial arch. This indicates that they play at least some unique roles in branchial arch development, although expression of both is enhanced by ET-1 signaling. *eHAND*-null embryos die early before eHAND's role in branchial arch formation can be defined (Firulli et al., 1998); tissue-specific gene targeting approaches should reveal if eHAND is important in branchial arch development and to what degree dHAND and eHAND overlap in their function.

The ET-1-dHAND-Msx1 pathway and the hypoplastic branchial arches seen in *dHAND*-null embryos suggest potential molecular models that might regulate branchial arch growth (Fig. 9). *Msx1* expression is associated with morphogenesis in numerous embryonic regions of inductive epithelial-mesenchymal interactions. For example, the epithelial-derived apical ectodermal ridge of the limb bud induces *Msx1* expression in the underlying mesenchyme (Wang and Sassoon, 1995); a similar process controls *Msx1* expression during tooth morphogenesis (Vainio et al., 1993). *Msx1* and *Msx2* are expressed in the distal branchial arch, similar to *dHAND*, in the subepithelial layer which is the leading edge of the growing arch. Msx2 has been implicated in mediating BMP-4-induced programmed cell death (Marazzi et al., 1997), while Msx1 may promote cellular proliferation (Song et al., 1992); a balance between the two may be critical to appropriate growth of the branchial arch. In contrast, the expression of *Dlx2*, which is thought to contribute to mesenchymal differentiation, overlaps *Msx* expression in the distal arch but extends to the proximal arch as well. Dlx2 forms nonfunctional heterodimers with Msx proteins in vitro, suggesting that a balance of Dlx2 and Msx proteins may be important in regulating the competing drives for cellular differentiation, proliferation and cell death during branchial arch growth (Zhang et al., 1996, 1997). By regulating *Msx1* expression, dHAND may mediate the transduction of epithelial signals such as ET-1 via the ET_A receptor in the mesenchymal portion of the branchial arch. Disruption of this signaling pathway results in increased apoptosis of mesenchymal cells of the branchial arch as seen in *dHAND*-null branchial arches. Similarly, dHAND appears to be mediating ET-1-initiated endothelial-mesenchymal interactions in the aortic arch arteries.

Elucidating the molecular pathways and mechanisms regulating branchial arch and aortic arch development is fundamental to understanding the pathogenesis of the numerous congenital syndromes involving the derivatives of these structures. These include Treacher-Collins syndrome (Sulik et al., 1987) and Pierre Robin sequence (Dennison, 1965), which manifest as abnormalities of first and second branchial arch derivatives. The spectrum of phenotypes encompassed by CATCH-22 syndrome are characterized by defects in derivatives of the third and fourth branchial arch and pouches, including craniofacial and aortic arch defects. Many of the typical defects represent hypoplasia or incomplete development of affected structures, suggestive of apoptotic or proliferative defects during development. It will be important to determine if mutations in any of the genes described here or factors upstream or downstream to them are involved in the pathogenesis of these neural-crest-related defects. In this sense, generating molecular pathways as we have begun to do here represents the first step in identifying disease causing genes and their mechanisms of action.

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