INTRODUCTION

Neural crest cells are a migratory population of cells that originate at the dorsal lip of the neural fold (Le Douarin et al., 1993; Bronner-Fraser, 1995). Once at their final destinations, they differentiate into a wide variety of derivatives, including epidermal melanocytes, neurons, endocrine and paraendocrine derivatives, and much of the bone, cartilage and connective tissue of the head and neck (Le Douarin, 1982; Noden, 1988). Migration, proliferation and differentiation of these cells are highly influenced by local environmental factors encountered during and after migration (Jessel and Melton, 1992; Le Douarin et al., 1993; Shah et al., 1996).

Crest cells that participate in craniofacial morphogenesis arise from the cephalic neural crest. Head development begins with cephalic neural crest cell migration from the posterior midbrain-hindbrain region into the pharyngeal arches in an axial level-specific pattern (Lumsden et al., 1991; Serbedzija et al., 1992; Kontges and Lumsden, 1996). Once there, the crest-derived ectomesenchyme undergoes inductive changes, resulting in the development of craniofacial bones and cartilages (Le Lievre and Le Douarin, 1975; Couly et al., 1993; Kontges and Lumsden, 1996). Interestingly, long-term fate mapping of cephalic neural crest cells has clearly shown a constrained pattern of cranial skeletomuscular connectivity with respect to the positional origin of the constitutive crest cells (Kontges and Lumsden, 1996). This may result from interaction of ectomesenchymal cells with paraxial mesoderm-derived cells that are segregated in the mesenchymal core of the arch (Trainor et al., 1994; Trainor and Tam, 1995), the anlage to the bulk of the musculature of the head and jaw. These two precursor cell types likely instruct each other to initiate the correct morphogenetic program. Development of neural crest cells within the pharyngeal arches relies upon the action of numerous transcription factors (Anderson, 1997). These factors guide migrating neural crest cells and later play a role in lineage determination, expansion and differentiation of neural crest derivatives. The first family of genes known to be involved in pharyngeal arch development were the Hox genes (Hunt et...
al., 1991). Specific Hox genes are expressed in arches 2-6, leading to the idea that a ‘Hox code’ was responsible for the development of all but the first (mandibular) arch, which developed by default programming. However, recent targeted mutations of transcription factors in mice have now shown that many genes are involved in the development of not only the first, but all of the pharyngeal arches. Mice with AP-2 (Schorle et al., 1996; Zhang et al., 1996), Cart1 (Zhao et al., 1996), Dlx-1 (Qiu et al., 1997), Dlx-2 (Qiu et al., 1995; Qiu et al., 1997), goosecoid (Rivera-Perez et al., 1995; Yamada et al., 1995), Mhox (Martin et al., 1995), Msx1 (Satokata and Maas, 1994), and Otx2 (Matsuo et al., 1995) null mutations, as well as RARαγ double knockouts (Lohnes et al., 1994), all suffer defects in cephalic neural crest-derived skeletal elements. These mutations often produce overlapping phenotypes, suggesting that multiple factors form a combinatorial code to pattern individual skeletal elements. However, evidence of an actual signaling pathway, including upstream intercellular activators, has not yet been found.

Patterning of specific regions of the developing heart also depends on a subset of cephalic neural crest cells, termed the cardiac neural crest (Kuratani and Kirby, 1991; Kirby, 1993; Kirby and Waldo, 1995). Cardiac neural crest ablation experiments illustrate that the development of aortic arch arteries and the conotruncal region of the heart rely upon contribution from crest cells. Mouse genes whose null mutations affect the cardiac neural crest and disrupt these structures include ActRIIB (Oh and Li, 1997), dHAND (Sravastava et al., 1997), HoxA3 (Chisaka and Capecchi, 1991), NF-1 (Brannan et al., 1994), NF-3 (Donovan et al., 1996) and Pax3 (Conway et al., 1997). RARαβ2, αβ2, αγγγ, γγ double mutants also exhibit related abnormalities (Mendelsohn et al., 1994). However, as in the cephalic neural crest, a distinct signaling pathway that might initiate an inductive developmental program has not been delineated.

One group of genes that may play a potential role in neural crest determination are the endothelins and their receptors. The endothelin (ET) pathway consists of three closely related small peptide ligands (ET-1, -2 and -3) that bind to one or both of the G protein-coupled endothelin receptors, ETA and ETB (Arai et al., 1990; Sakurai et al., 1990; Yanagisawa, 1994). Recent evidence shows that endothelins and their receptors are required for development of specific subsets of neural crest-derived tissues: e.g. the disruption of the ET-1 gene causes malformations in pharyngeal arch-derived structures and the heart (Kurihara et al., 1994), while mice deficient in either ET-3 or ETB develop white spotted coats and aganglionic megacolon due to the absence of neural crest-derived melanocytes and enteric neurons (Baynash et al., 1994; Hosoda et al., 1994). The phenotype observed in ET-3/ETB deficient mice does not overlap with that of ET-1 deficient animals, indicating that the developmental effect of ET-1 is not mediated by the ETB receptor.

To define the developmental role of the ETA receptor, we generated ETα-null mice by gene targeting. ETα-/- mice are born alive but suffer severe craniofacial and cardiovascular defects, similar to those observed in ET-1 deficient mice, and die soon after birth. Examination of expression patterns of ETA and ET-1 during development suggests that ET-1/ETA interaction defines a novel signaling pathway crucial for pharyngeal arch development. This pathway includes goosecoid, whose expression in the pharyngeal arches depends on ETA signaling.

MATERIALS AND METHODS

Gene targeting

The targeting construct was designed to replace exons 5 and 6 of the ETA gene, which corresponds to the sixth and seventh transmembrane domains of this G protein-coupled receptor. Homologous sequences for the ETA gene were obtained from an EMBL3 mouse genomic library (Clontech), and were composed of a 12-κb SalI/Sall fragment 5' to exons 5 and 6 and a 1.2-κb SacI/Sphi fragment 3' to exons 5 and 6. A universal neo-TK template plasmid vector (Hosoda et al., 1994), which contains a neomycin gene cassette driven by the RNA polymerase II promoter as well as two tandem herpes simplex virus thymidine kinase cassette, was used to construct the targeting vector. The targeting construct was electroporated into JH-1 ES cells (Hosoda et al., 1994) maintained on SNL76/7 fibroblast feeder layers (a gift from A. Bradley). Following selection with G418 and FIAU, surviving colonies were screened with three probes (see Fig. 1) to confirm homologous recombination by Southern blot analysis. Probes A and C were used following digestion of genomic DNA with XbaI, while probe B was used following digestion with EcoRI. Correctly targeted ES cells clones were injected into blastocysts from C57BL/6 mice to obtain chimeras, which transmitted the targeted allele in their germline. Genotypes of mice were confirmed using probe C. To establish the deletion on an inbred background, mice were bred with 129SvEv mice. Subsequent genotyping was performed by the polymerase chain reaction (PCR) using genomic DNA isolated from tail biopsies. Primers used to detect the mutant allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (neo) and 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette). Primers used to detect the wild-type allele were 5'-TGGGATGAGTCCTCTGAGGAC-3' (3' to the neo cassette).

Radioligand binding assay

Skin from ETα+/- and ETα-/- E18.5 embryos were minced and seeded onto the bottom of a 25 cm2 flask in Dulbecco's modified Eagle's medium (DMEM) plus 20% fetal calf serum. Outgrowths of mesenchymal cells were passaged every 3 days and passage five cells were used in the assay. The binding assay was performed as previously described (Sakurai et al., 1990), using 125I-ET-1 (2,000 Ci/mmol; Amersham) as the tracer. Unlabeled ET-1/ET A antagonist, FR139317 (Fujisawa Pharmaceutical), an ET A antagonist, was used as competitors. Non-specific binding was determined in the presence of 1x10^-7 unlabel ET-1. The ratio of non-specific to specific binding in the absence of competitors was approximately 2% and 16% in the cell lines from the heterozygous and homozygous animals, respectively.

Respiratory responses

E18.5 embryos were delivered by Caesarean section and immediately tracheostomized with a polyethylene tube (SP8, Natsune, Tokyo) as previously described (Kuwaki et al., 1996). Mice were then placed in a warmed chamber (32-35°C) for at least 30 minutes before ventilation was measured. Mice were placed in a plastic chamber, where they initially breathed room air. They were then exposed to hypoxic (1:1 air:No2) or hyperoxic (5% CO2—95% O2) gas mixtures. During this time, PaCO2 and PaO2 in the chamber were continuously monitored (Respira H26, San-Ei-Instruments). When the gas within the chamber reached equilibrium, ventilatory measurements were performed for 3-
5 minutes. Pressure within the chamber was measured with a
transducer (Model TP603T, Nihon Kohden), amplified (Model
AR601G, Nihon Kohden) and stored in a data recorder (Model XR-
7000L, TEAC). After analog to digital conversion (MP100, Biopac
Systems), the data was fed into a Macintosh computer for computation
as previously described (Kuwaki et al., 1996).

Histology and in situ hybridizations
For routine analysis, embryos were fixed in Bouin’s fixative,
embedded and sectioned at 4 μm. Paraffin sections of embryos were
stained with hematoxylin and eosin as previously described (Hosoda
et al., 1994). For skeleton analysis, E18.5 embryos were collected,
prepared and stained with alizarin red and alcian blue to examine bone
and cartilage formation, respectively (Kochhar, 1973). Cartilaginous
fetal skeletons (E12.5-E14.5) were prepared and
stained with alcian blue (Jegalian and De Robertis,
1992). For in situ hybridizations, embryos were
collected and fixed in 4% paraformaldehyde. Sectional
in situ hybridizations were performed as described previously (Benjamin et al., 1997) except that riboprobes were labeled with both 35S-CTP and 35S-
UTP (Amersham) using the Maxiscript In Vitro Translation Kit (Ambion). The ET A probe was a 350 bp BamHI-EcoRI fragment and the ET-1 probe was a
380 bp SacI-HindIII fragment, both from mouse
cDNAs. Whole-mount in situ hybridizations were
performed using E10.5 embryos as previously
described (Wilkinson, 1992) using digoxigenin-
labeled probes for Dlx-1 (McGuinness et al., 1996)
and goosecoid (Shawlot and Behringer, 1995). Embryos were genotyped by PCR using genomic
DNA isolated from yolk sac.

Whole-mount immunohistochemistry
The immunohistochemistry protocol used was adapted
from that of Davis et al., (1991). E10.5 and E11.5
embryos were collected and fixed in 4% paraformaldehyde at 4°C overnight. After rinsing in PBS, they were dehydrated through a graded series of
methanols, bleached in 5% hydrogen peroxide in
methanol for 5 hours and rinsed in 100% methanol.
After rehydrating into PBS, embryos were blocked for
1 hour in 0.5% Triton X-100 and 2% skimmed milk
powder in PBS (PBSMT) and then incubated
overnight at 4°C with an anti-NF160 antibody (Sigma,
#N5264) at a dilution of 1:100 in PBSMT. After
rinsing 5 times for 1 hour each in PBSMT, embryos
were incubated overnight at 4°C with a horseradish
peroxidase-conjugated goat anti-mouse IgG antibody
(Sigma, #A3682) at a dilution of 1:200 in PBSMT.
After rinsing 5 times for 1 hour each in PBSMT, embryos
were incubated in DAB using the Liquid DAB
Substrate Kit (Zymed) and following the
manufacturer’s instructions. After rinsing in PBT twice for 10 minutes each, embryos were dehydrated
through a graded methanol series and cleared in 2:1
benzyl benzoate/benzyl alcohol.

RESULTS
Developmental patterns of ET A and ET-1
expression in pharyngeal arches
Targeted disruption of the ET-1 gene in mice
results in craniofacial and cardiovascular defects (Kurihara et
al., 1994) that are not observed in ET B−/− mice (Hosoda et al.,
1994). These defects are therefore probably not a result of loss
of ET B signaling. The present study was designed to examine
the role of the other known endothelin receptor, the ET A
receptor, in development of these tissues. In situ hybridization
analysis found that cephalic crest cells become ET A-positive
as soon as they leave the rhombomeres, while the neural tube
itself does not express ET A (data not shown). At E9.5, ET A
mRNA was observed in migrating cephalic neural crest cells
extending from the hindbrain into the pharyngeal arches (Fig.
1A). Message was also present in the facio-acoustic neural
crest complex (Fig. 1A). The mesenchyme of pharyngeal

![Fig. 1. In situ hybridization analysis of ET A (A, C, E, G) and ET-1 (B, D, F, H)
transcripts in the pharyngeal arch region of E9.5 wild-type embryos. (A, B) Sections through the sagittal plane. ET A mRNA is detected in head and first
pharyngeal arch (pa1) mesenchyme, as well as in the facio-acoustic neural crest complex (+). ET-1 mRNA is restricted to the epithelium of the arch and the
endoderm of pharyngeal pouches (pp) 1 and 2. (C-F) Sections through the transverse plane. (C, E) ET A mRNA is observed in the mesenchyme of the head and
pharyngeal arches 1, 2 and 3. (D) ET-1 mRNA is found on the arch epithelium.
(F) ET-1 mRNA is found in the endothelium of the communication between
pharyngeal arch artery 3 and the dorsal aorta (arrow), and in the paraxial mesoderm
core of arch 2 (arrowhead). (G, H) Sections through the frontal plane. The asterisks
in (H) mark ET-1 message in the pharyngeal pouches. ov, otic vesicle.](image-url)
arches 1, 2 and 3 were also ET\(_A\)-positive, although the arch epithelium was negative (Fig. 1A,C,E,G). It is noteworthy that in Fig. 1E, ET\(_A\) message is less abundant in the core of arch 2. ET\(_A\) mRNA was also observed in head and body mesenchyme (Fig. 1A,C,E,G).

Conversely, ET-1 message was confined to the ectodermal epithelium of arches 1, 2 and 3 and their associated endodermal pouch epithelia (Fig. 1B,D,F). Interestingly, ET-1 mRNA was also observed in the paraxial mesoderm-derived core of arches 1 (data not shown) and 2 (Fig. 1F). Expression of ET-1 in paraxial mesoderm, which gives rise to most of the muscles and vasculature of the head, has been documented before, but was interpreted as being simply arch mesenchyme staining (Maemura et al., 1996). As mentioned above, paraxial mesoderm does not mix with neural crest cells in the pharyngeal arches, but rather colonizes only the core mesenchyme (Trainor and Tam, 1995). Since neural crest cells overlay the mesoderm, it is likely that ET-1 in the mesoderm, together with ET-1 in the arch epithelium, acts on the ectomesenchymal cells to help further control craniofacial morphogenesis.

**Expression of ET\(_A\) and ET-1 in the developing heart**

In situ hybridization analysis of the heart in E8.5 (data not shown) and E9.5 (Fig. 7E) wild-type embryos found abundant ET\(_A\) mRNA in the myocardium of the ventricle, atrium and bulbus cordis, as well as in the mesenchyme of the aortic arches. Conversely, ET-1 expression was observed in the endocardium of the heart chambers and the endothelial lining of the arch arteries (Fig. 7F). This suggests that local interactions between cardiac neural crest-derived mesenchyme and the underlying endothelium of the arches results in initiation or continuation of a cardiac developmental program (see below).

**Targeted disruption of the ET\(_A\) gene**

To disrupt the ET\(_A\) gene, we constructed a targeting vector that would replace exons 5 and 6, which encode the sixth and seventh transmembrane domains, with a neomycin resistance cassette by homologous recombination. Two thymidine kinase (TK) genes were included in tandem at the 3' end of the targeting vector as a negative selection marker for non-homologous recombination events (Fig. 2A). The targeting construct was electroporated into ES cells, and following selection for homologous recombination, surviving clones were screened by Southern blot (Fig. 2B). Correctly targeted clones were used to generate chimeric mice by blastocyst injection as described in Materials and Methods. Germline

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**Fig. 2.** Disruption of the ET\(_A\) gene. (A) Strategy for disruption of the ET\(_A\) gene. A partial map of the wild-type mouse ET\(_A\) gene, including exons 5 and 6, which encode the sixth and seventh transmembrane domains, respectively, is shown. Southern blot probes and PCR primers used are shown below the map of the targeted allele. Restriction enzymes: E, EcoRI; Sc, SacI; Sl, SalI; Sp, SpeI; X, XbaI. (B) Southern blot analysis of DNA from wild-type and ET\(_A\) mutant ES cells. Genomic DNA was digested with XbaI and the blot hybridized with the 5' probe (probe A), the internal probe (probe B) or the 3' probe (probe C). Wild-type (8.5 kb) and targeted (2.4, 4.5 or 7.0 kb) fragments are indicated. (C) Southern blot analysis of tail DNA from wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mutant mice. The DNA was digested with XbaI and the blot hybridized with probe C. (D) Radioligand binding assay for the ET\(_A\) receptor protein. Unlabeled ET-1, FR139317 (an ET\(_A\) antagonist) or IRL1620 (an ET\(_B\) agonist) were used as competitors. Open and closed symbols indicate ET\(_A\)^{+/+} and ET\(_A\)^{-/-} cells, respectively.
transmission of the disrupted allele resulted in heterozygous mice that were healthy and fertile. The number of homozygous mutant mice obtained from heterozygous crosses (80/277; 29%) was close to the expected Mendelian frequency, indicating that homozygous embryos were viable up to parturition (Fig. 2C).

To demonstrate that the ETA targeted allele is functionally null, we used fibroblasts derived from heterozygous (ETA<sup>+/−</sup>) and homozygous (ETA<sup>−/−</sup>) mutant E18.5 embryos in a competitive radioligand binding assay (Fig. 2D). In ETA<sup>+/−</sup> cells, we detected a large amount of specific 125I-ET-1 binding that was displaceable by an ETA-selective synthetic ligand (FR139317) but not by an ETB-selective ligand (IRL1620). The specific binding sensitive to the ETA antagonist was absent in ETA<sup>−/−</sup> cells, demonstrating lack of functional ETA receptors. A 125I-ET-1 binding assay performed on membrane preparations from whole near-term embryos gave similar results (data not shown).

**Respiratory defects in neonates**

At birth, ETA<sup>−/−</sup> pups showed several striking characteristics that are virtually identical to those observed in mice deficient either for ET-1 (Kurihara et al., 1994) or endothelin converting enzyme-1 (ECE-1; Yanagisawa et al., 1998) (Fig. 3A). Most striking was the poorly formed mandible that sometimes lacked a midline fusion. Mutant pups also had hypoplastic pinnae and a sunken ventral neck. They were cyanotic, had gasping breathing movements, and died within 30 minutes of birth. E18.5 mutant embryos delivered by Caesarean section followed by tracheostomy survived for more than 25 hours, indicating a structural defect in the upper airway as the primary cause of death. By alleviating mechanical asphyxia in P0 ETA<sup>−/−</sup> pups, we were also able to investigate the central ventilatory responses to atmospheric hypoxia and hypercapnia, an important aspect considering the severe impairment of responses previously observed in P0 ET-1<sup>−/−</sup> pups (Kuwaki et al., 1996). When P0 wild-type and ETA<sup>−/−</sup> pups breathed room air, the respiratory minute volume was not significantly different between the two groups. However, when pups breathed hypoxic gas (1:1 room air:N<sub>2</sub>), ventilatory responses were significantly decreased 17.6% in ETA<sup>−/−</sup> mice, compared with an increase of 16.6% in wild-type pups. Similarly, when wild-type and mutant pups breathed hypercapnic gas (5% CO<sub>2</sub>-95% O<sub>2</sub>), mutant pups showed a decreased breathing response of 8.4%, while the wild-type breathing response markedly increased by 64.0%. Since both the ETA receptor and ET-1 are found in central nervous system areas that participate in respiratory control (Hori et al., 1992), these current results strongly suggest that ET-1/ETA-mediated signaling is important in the central control of respiration after birth.

**Abnormalities in craniofacial morphogenesis**

The physical appearance of mutant pups was indicative of defects in cephalic neural crest derivatives. Thus, we further analyzed the development of individual structures derived from the pharyngeal arches. Meckel’s cartilage, the first arch cartilage, was present in E14.5 wild-type (ETA<sup>+/+</sup>) embryos (Fig. 4A), but was absent in ETA<sup>−/−</sup> embryos (Fig. 4B). The cartilaginous rudiment of the hyoid bone (derived from Reichert’s cartilage, the second arch cartilage) was evident in wild-type and ETA<sup>−/−</sup> embryos by E14.5 (Fig. 4A,B), but in the latter it was moved ventrorostally and fused to a cartilaginous precursor of a cranial bone, forming a ring-like structure. Analysis of E18.5 embryos revealed that this fusion occurred between the lesser horns of the hyoid bone and an area encompassing the basisphenoid bone, the pterygoid bones and the ala temporalis cartilage (Fig. 4D,F). The mandibular bone was hypoplastic, highly disorganized and abnormally articulated with the jugal bone of the zygomatic arch, which itself was smaller than normal (Fig. 4D). Furthermore, aberrant membranous bone extended ventrocaudally from the mandible, forming a disorganized sheet-like structure that fused to endochondral bone near the anterior edge of the basisphenoid bone (Fig. 4D). These fusions resulted in a severe constriction of the upper airway (Fig. 4F), likely contributing to the observed mechanical asphyxia. These abnormalities were exaggerated by the fusion of the soft palate to the lateral floor of the oral cavity proximal to the squamous/respiratory epithelial boundary, and by a thickening of the palate, essentially preventing oral respiration (Fig. 4H). Further, the tongue and associated muscles were...
severely hypoplastic (Fig. 4J), the alisphenoid, palatine, pterygoid and squamosal bones underdeveloped, many of the mandibular salivary and submandibular glands absent, and the thymus hypoplastic and rostrally displaced (data not shown). The penetrance of each of these defects was 100% (14/14 embryos examined). Thus, multiple structures derived from the neural crest component of the first three pharyngeal arches were disrupted in ETA−/− embryos.

**Aberrant middle ear development**

Abnormalities of neural crest-derived structures in the middle ear were also observed in 100% (14/14) of ETA−/− embryos examined. The malleus and incus, as well as the tympanic ring and the cartilaginous anlage of the styloid process, were absent (Fig. 5B), although vestiges of these structures were observed in histological sections (Fig. 5D). This suggests that the rudimentary structures that do form lack normal articulations and are subsequently lost during skeleton staining. The gonial bone was also absent and the cartilaginous rudiment of the third ossicle, the stapes, was absent in 79% (11/14) of mutant embryos (Fig. 5B). The tubotympanic recess, formed by the elongation of the endodermal lining of the first pharyngeal pouch, and the external auditory meatus, formed by the ingrowth of epithelial ectodermal cells, were also absent, and consequentially, so too was the tympanic membrane (Fig. 5D). In situ hybridization analysis clearly shows expression of ET-1 in the pharyngeal pouches at E9.5 (see Fig. 1D,F), suggesting that loss of the tubotympanic recess is a direct result of loss of ETA signaling. However, ET-1 expression is not observed in the epithelial layers around the future external auditory meatus. Its loss is more likely a result of the loss of the tympanic ring, since the tympanic ring primordium is believed to induce early epithelial invagination (Mallo and Gridley, 1996). The inner ear of E18.5 homozygous mutants appeared normal (data not shown).

**Defects in distal branches of the trigeminal and facial nerves**

Cephalic neural crest cells also contribute to the development of the peripheral nervous system of the head, including the trigeminal, facial and glossopharyngeal ganglia and associated nerves (Le Douarin, 1982). As shown in Fig. 1A, abundant ETA message is associated with the facial/acoustic neural crest complex at E9.5. This indicates that ETA signaling may also be important in neuronal development. To investigate this possibility, we performed whole-mount immunohistochemical analysis of neurofilament expression in E10.5 and E11.5 embryos using an anti-NF160 antibody. Examination at low magnification revealed no changes in the peripheral nervous system of ETA−/− embryos outside the pharyngeal arches. Further, at both ages, the spatial configuration of the

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**Fig. 4.** Analysis of craniofacial defects in ETA−/− mice.

(A-D) Skeleton preparations of ETA+/+ (A, C) and ETA−/− (B, D) embryos. (A, B) Lateral view of E14.5 littermate skulls (alcoholic blue). In the mutant, Meckel’s cartilage (mc) is absent. The arrowhead points to an abnormal cartilaginous body and the asterisk marks the site of fusion between the cartilaginous precursor of the hyoid (h) bone and the cartilaginous anlage of one or more bones at the base of the skull. (C, D) V entral view of E18.5 embryo skulls from littermates (alizarin red and alci

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ep, epiglottis; in, incisor; nc, nasal cavity; th, thyroid cartilage.
trigeminal, facial and glossopharyngeal ganglia of \( ET_A^{+/−} \) embryos appeared normal (Fig. 6B,D). However, while the mandibular branch of the trigeminal nerve and the facial nerve correctly innervated the mandibular and second arches, respectively, the nerves failed to project to the most distal aspects of the \( ET_A^{+/−} \)-arches (Fig. 6B,D). Further, ectopic fiber growth was observed on both the mandibular branch of the trigeminal nerve (Fig. 6D) and the facial nerve (data not shown), whereas the maxillary branch of the trigeminal nerve, which innervates the maxillary portion of the first arch, showed decreased arborization within the frontonasal region (Fig. 6D). These results show that absence of \( ETA \) signaling does not affect the patterning of cranial ganglia, although the distal ends of several nerves emanating from these ganglia are abnormal.

Abnormal cardiac and outflow tract development

There were also numerous abnormalities in the heart and outflow tract of E18.5 \( ET_A^{-/−} \) embryos, with a cumulative penetrance of 100%. A common defect observed in the outflow tract was interruption of the aorta (44% occurrence; 7/16 P0 \( ET_A^{-/−} \) pups examined suffered this defect), which resulted in a dominant ductus arteriosus that subsequently joined the dorsal aorta (Fig. 7B). Also observed in \( ET_A^{-/−} \) embryos were tubular hypoplasia (56%; 9/16), absent right subclavian artery (44%; 4/9), extra arteries branching off the right and left common carotid arteries (23%; 2/9), and right dorsal aorta with right-sided ductus arteriosus (11%; 1/9) (Fig. 7B). A subpopulation of neural crest cells in pharyngeal arches 3, 4 and 6 migrate to the cardiac outflow tract and conotruncal regions, where they are involved in maturation of the great
arteries and outflow septation complex (Kirby and Waldo, 1995). Our observation that fourth arch artery derivatives (i.e. a segment of the aortic arch between the left common carotid artery and the left subclavian artery, as well as the proximal right subclavian artery) are most profoundly affected in $ET_A^{-/-}$ (this study) and $ECE-1^{-/-}$ mice (Yanagisawa et al., 1998) suggests that cardiac neural crest cells cannot correctly pattern the aortic arch vessels without ETA receptor-mediated signaling.

We also observed septation and alignment defects in $ET_A^{-/-}$ embryos. Ventricular septal defect (VSD) was observed in 92% (23/25) of E18.5 homozygous mutant embryos (Fig. 7D). The aorta also frequently overrode the defective septum (44%; 11/25). Double outlet-right ventricle (DORV) (28%; 7/25), persistent truncus arteriosus (PTA) (6%; 1/16) and complete transposition of the great arteries (TGA) (13%; 2/16) (Fig. 7D) were also detected. Similar defects have been reported in $ET-1^{-/-}$ embryos, but only following administration of anti-ET-1 neutralizing antibodies or ETA antagonists to pregnant ET-1+/− females (Kurihara et al., 1995), illustrating that the effects observed in the present study are a result of loss of ET-1/ETA interactions.

Previous studies in avian systems showed that ablation of the cardiac neural crest results in a highly similar repertoire of phenotypes (Kirby, 1993), strongly suggesting that ETA receptor disruption affects either the migration or subsequent proliferation/differentiation of cardiac neural crest cells.

**Expression of transcription factors in the pharyngeal arches of $ET_A^{-/-}$ embryos**

Disruption of cephalic neural crest derivatives in $ET_A^{-/-}$ embryos suggests that ETA-mediated signaling plays a crucial role in the inductive processes that accompany head development. The complementary expression patterns of ETA and ET-1 suggest that these molecules affect neural crest development through local interactions within the pharyngeal arch ectomesenchyme, rather than affecting the migration of neural crest cells. Therefore, we examined the expression of two genes known to be expressed in the pharyngeal arches by postmigratory neural crest cells, $Dlx-1$ (Price et al., 1991) and $goosecoid$ (Blum et al., 1992). These genes are both expressed in the first and second arches at E10.5, although their spatiotemporal expression patterns (Dolle et al., 1992; Gaunt et al., 1993; Qiu et al., 1997) and knockout phenotypes indicate that they are each involved in the development of unique subsets of cephalic neural crest cell derivatives. $Dlx-1$-null mice have defects in the ala temporalis, with partial penetrance of an abnormal phenotype in the stapes, styloid process, and palatine and pterygoid bones (Qiu et al., 1997). Conversely, $goosecoid$-null embryos show defects in multiple craniofacial structures, including the alisphenoid, pterygoid, palatine, tympanic, maxillary, frontal and mandibular bones (Rivera-Perez et al., 1995; Yamada et al., 1995).

Using whole-mount in situ hybridization analysis, $Dlx-1$ expression in E10.5 $ET_A^{-/-}$ embryos was observed over the lateral aspects of the first mandibular arch and second arch, a
pattern that did not change in ET_A<sup>−/−</sup> embryos. goosecoid expression in E10.5 ET_A<sup>+/−</sup> embryos was observed in the medial aspects of both the posterior half of the first mandibular arch and the anterior half of the second arch, as well as in the nasal pits and limb buds (Fig. 8A). While goosecoid expression was not affected in the nasal pits and limb buds of E10.5 ET_A<sup>−/−</sup> embryos, expression in arches one and two was undetectable. These findings indicate either that expression of goosecoid in ectomesenchymal cells requires ET_A<sup>−</sup>-mediated signaling, or that the subpopulation of ectomesenchymal cells that normally express goosecoid is absent in ET_A<sup>−/−</sup> embryos.

**DISCUSSION**

We inactivated the ET_A gene by targeted deletion of two of the seven transmembrane spanning domains of this G protein-coupled receptor. Homozygous mutant mice exhibit multiple defects in cephalic neural crest cell derivatives. These defects appear to result from loss of inductive signals, both within the pharyngeal arches and their associated arch arteries. Thus, ET_A-mediated signaling appears to define a novel pathway crucial for cephalic neural crest development, and along with the 5-HT2B receptor (Choi et al., 1997), the ET_B receptor (Hosoda et al., 1994), ET-3 (Baynash et al., 1994) and Gα<sub>13</sub> (Offermanns et al., 1997), illustrate the importance of G protein-coupled signaling pathways in embryonic development. Although ET-1 can interact with both ET_A and ET_B receptors with high affinities, the ET-1/ET_A axis does not overlap with the ET-3/ET_B pathway (Baynash et al., 1994; Hosoda et al., 1994), as the enteric neurons of the distal colon and epidermal/choroidal melanocytes were both normal in ET_A<sup>−/−</sup> mice (data not shown), and defects described in this study were not seen in ET-3 or ET_B-deficient animals (Baynash et al., 1994; Hosoda et al., 1994).

**Epithelio-ectomesenchymal and paraxial mesodermo-ectomesenchymal interactions mediated by the ET-1/ET_A pathway**

We have shown in this study that the spatial expression patterns of ET-1 and ET_A in the pharyngeal arches are complementary to each other and provide a cellular basis for the changes observed in gene expression within the arches of ET_A<sup>−/−</sup> embryos (Fig. 8B). Reciprocal expression of extracellular ligands and their cognate cell surface receptors often plays a major role in initiating or maintaining morphogenetic changes of developing structures during epithelial/mesenchymal interactions (Andermarcher et al., 1996; Robertson and Mason, 1997). The interaction of ET-1 with the ET_A receptor is likely aided by the spatial distribution of neural crest cells within the arches (Trainor and Tam, 1995). The presence of cephalic neural crest cells just below the surface ectoderm would allow ET-1 expressed by the epithelial cells to act on the ectomesenchymal cells during epithelial/mesenchymal interactions, initiating ET_A signaling. Further regulation of ectomesenchymal development may be imparted by the paraxial mesoderm located in the core of the arch. Neural crest cells and paraxial mesoderm cells are spatially segregated during arch development, possibly allowing paraxial mesoderm to instruct the ectomesenchyme in specific aspects of arch development through interaction of ET-1 with the ET_A receptor. Such action of the mesoderm on the ectomesenchyme may help explain the association of specific arch-derived muscles with skeletal derivatives of the same arch (Kontges and Lumsden, 1996). It is also possible that the

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**Fig. 8.** (A) Whole-mount in situ hybridization of ET_A<sup>+/−</sup> and ET_A<sup>−/−</sup> E10.5 embryos using Dlx-1 and goosecoid (gsc) riboprobes. Genotype of embryos is indicated, as are pharyngeal arches 1 and 2. While Dlx-1 and goosecoid are both expressed in heterozygous embryos, goosecoid expression is absent in homozygous mutant embryos. (B) Proposed model of craniofacial morphogenesis. The interaction of ET-1 (expressed in the epithelium and paraxial mesoderm core of the pharyngeal arches) with the ET_A receptor (expressed by the neural crest-derived ectomesenchymal cells) initiates a signaling cascade that likely includes activation of numerous transcription factors including goosecoid. This results in the proper developmental programming of neural crest derivatives in the jaw and neck region. All skeletal elements shown on the right are affected by the ET_A mutation. al, alisphenoid.
ectomesenchyme, having been stimulated by ET-1 from the mesoderm, acts in a reciprocal manner and produces factors that directly influence mesoderm development in a manner similar to the positive feedback loop involving Sonic hedgehog and FGF-4 expression during limb development (Laufer et al., 1994; Niswander et al., 1994). The close proximity of the crest cells with the ectodermal epithelium and paraxial mesoderm is important in this model, as mature ET-1 appears to act over only short distances during development (Yanagisawa et al., 1998).

**ET A-mediated signaling and craniofacial development**

The ET A mutation results in defects in both ectomesenchymal and neuronal derivatives. While the ectomesenchymal defects clearly seem to be a direct result of loss of ET A signaling, the subtle defects in neurogenic neural crest derivatives could be secondary to other more prominent arch abnormalities, including loss of guidance or growth factors within the pharyngeal arches necessary for distal nerve projection. An elegant scheme of the migration patterns of cephalic neural crest cell subpopulations has been generated through long-term fate mapping (Lumsden et al., 1991; Serbedzija et al., 1992; Coulby et al., 1993; Kontges and Lumsden, 1996), and it is possible to use these maps to delineate which crest subpopulations are affected in mice containing targeted gene disruptions (Kontges and Lumsden, 1996). The defects observed in ET A−/− embryos indicate that the mutation disrupts the development of cells arising from the posterior midbrain as well as rhombomeres 1, 2, 4 and 6. This suggests that ET A signaling is required for either initial neural crest migration or in the subsequent maintenance/differentiation of the ectomesenchymal derivatives. Preliminary in situ hybridization analysis has revealed that initial neural crest cell migration appears unaffected in ET A−/− embryos (D.E. Clouthier et al., unpublished). This then indicates that the ET A mutation results in absence of a signaling pathway that is crucial during further differentiation/development of neural crest derivatives within the arches, likely during epithelial/ectomesenchymal and paraxial mesodermal/ectomesenchymal interactions (Fig. 7B).

**goosecoid is a potential downstream effector of ET A signaling**

An intriguing finding of this study is that goosecoid expression is absent within the pharyngeal arches of E10.5 ET A−/− embryos. This suggests either that ET A signaling directly or indirectly induces goosecoid expression within the neural crest-derived ectomesenchyme, or that the subset of ectomesenchymal cells that normally express goosecoid are absent in ET A−/− embryos. Based on the expression patterns of several other neural crest markers (D.E. Clouthier et al., unpublished), we believe that the former explanation is more plausible, making goosecoid a probable downstream factor in an ET A receptor signaling pathway. Targeted disruption of goosecoid in mice affects similar cell populations to those in ET A−/− embryos, although the phenotype is less severe (Rivera-Perez et al., 1995; Yamada et al., 1995). This suggests that goosecoid is not the only downstream factor that is disrupted in ET A mutant embryos. On the other hand, while Dlx-1 expression is not affected in ET A-deficient embryos, both ET A-null and Dlx-1-null mouse share defects in several skull elements. Thus, Dlx-1 expression may affect similar downstream targets to ET A signaling, or function upstream of ET A receptor signaling.

**Lack of ET A signaling disrupts cardiac neural crest development**

While patterning of the outflow tract relies on an extensive bilateral and asymmetrical remodeling of arch arteries (Kirby and Waldo, 1995), the molecular mechanisms that regulate this process are poorly understood (Olson and Srivastava, 1996). This report shows that ET A−/− embryos have defects in great vessel alignment and development of the outflow tract. This effect is most certainly ET-1-mediated, as similar phenotypes are observed in ET-1−/− embryos after treatment of ET-1−/− pregant females with neutralizing antibodies against ET-1 or antagonists of the ET A receptor (Kurihara et al., 1995). Moreover, ECE-1−/− embryos, in which the levels of mature ET-1 are reduced (Yanagisawa et al., 1998), exhibit virtually identical cardiac defects. These findings are consistent with disruption of cardiac neural crest development, either during migration of the neural crest cells or their subsequent transition from ectomesenchymal cells into the smooth muscle and connective tissues of the arch arteries and septum (Kirby, 1993). Based on the expression patterns of ET A and ET-1 in the developing arch arteries, we suggest that ET-1 expression in the underlying endothelium of the arch arteries provides a microenvironmental signal for the neural crest derived mesenchymal cells of the arch arteries through the ET A receptor, resulting in the initiation or continuation of arch artery remodeling.

Defects in arch artery development have also been observed following targeted deletion of several others genes in mice, including RAR isofoms (Mendelsohn et al., 1994), NF-1 (Brannan et al., 1994), NT-3 (Donovan et al., 1996), Pax3 (Conway et al., 1997) and dHAND (Srivastava et al., 1997). Whether any of these genes are involved in the ET A-mediated signaling pathway is unknown. Interestingly, however, both dHAND (Srivastava et al., 1995) and the related gene eHAND (Cserjesi et al., 1995) are highly expressed within the pharyngeal arches around E9.5, suggesting that they also play a role in craniofacial development. The fact that ET A, dHAND and eHAND are expressed in two neural crest derived environments during development are further suggestive of a functional relationship.

**ET A and goosecoid mutations may cause human craniofacial defects**

The phenotype of ET A−/− mice resembles a spectrum of human conditions that are collectively termed CATCH 22 (cardiac anomaly, abnormal face, thymic hypoplasia, gieft palate, hypocalcemia, and chromosome 22 deletions) (Wilson et al., 1993) and velocardiofacial syndrome (Shprintzen et al., 1978; Goldberg et al., 1993). Both of these syndromes include numerous cardiac and craniofacial dysmorphisms. While none of the components of the endothelin pathway map to the chromosomal region deleted in CATCH 22 patients (22q11.2), the deletion could affect other components of the same signaling pathway. Indeed, a goosecoid-like homeobox gene (GSCL) expressed in early human development has recently been mapped within the CATCH 22 minimal critical region (Gottlieb et al., 1997). The phenotypes of ET A−/− and goosecoid−/− mice may help explain how a deletion of a
REFERENCES


