Recombinase-mediated cassette exchange reveals the selective use of $G_q/G_{11}$-dependent and -independent endothelin 1/endothelin type A receptor signaling in pharyngeal arch development

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The endothelin (Edn) system comprises three ligands (Edn1, Edn2 and Edn3) and their G-protein-coupled type A (Ednra) and type B (Ednrb) receptors. During embryogenesis, the Edn1/Ednra signaling is thought to regulate the dorsoventral axis patterning of pharyngeal arches via Dlx5/Dlx6 upregulation. To further clarify the underlying mechanism, we have established mice in which gene cassettes can be efficiently knocked-in into the Ednra locus using recombinase-mediated cassette exchange (RMCE) based on the Cre-lox system. The first homologous recombination introducing mutant $lox$-flanked Neo resulted in homeotic transformation of the lower jaw to an upper jaw, as expected. Subsequent RMCE-mediated knock-in of lacZ targeted its expression to the cranial/cardiac neural crest derivatives as well as in mesoderm-derived head mesenchyme. Knock-in of Ednra cDNA resulted in a complete rescue of craniofacial defects of Ednra-null mutants. By contrast, Ednrb cDNA could not rescue them except for the most distal pharyngeal structures. At early stages, the expression of Dlx5, Dlx6 and their downstream genes was downregulated and apoptotic cells distributed distally in the mandible of Ednrb-knock-in embryos. These results, together with similarity in craniofacial defects between Ednrb-knock-in mice and neural-crest-specific $G_q/G_{11}$-deficient mice, indicate that the dorsoventral axis patterning of pharyngeal arches is regulated by the Ednra-selective, $G_q/G_{11}$-dependent signaling, while the formation of the distal pharyngeal region is under the control of a $G_q/G_{11}$-independent signaling, which can be substituted by Ednrb. This RMCE-mediated knock-in system can serve as a useful tool for studies on gene functions in craniofacial development.

KEY WORDS: Endothelin, G protein-coupled receptor, Pharyngeal arch, Neural Crest, Mouse

INTRODUCTION

Craniofacial development in vertebrates is a complex process involving the coordinated interaction of different cell populations. During neural tube formation, cranial neural crest cells originate at the border between the neural plate and the surface ectoderm, and migrate ventrolaterally to contribute to the head mesenchyme together with mesodermal cells (Chai and Maxson, Jr, 2006; Le Douarin and Kalcheim, 1999; Noden and Trainor, 2005). The pharyngeal arches, the segmental structures of the embryonic head covered by ectoderm- and endoderm-derived epithelium, are populated with neural crest cells from the hindbrain and caudal midbrain, where they surround a mesodermal core. Within each pharyngeal arch, complex cellular interactions are thought to determine their regional identity and cell fates (Le Douarin and Kalcheim, 1999; Noden and Trainor, 2005).

In the two anterior pharyngeal arches, the first and second arches, dorsoventral axis patterning predominates the appropriate formation of chondrocranial elements and associated dental bones (Kontges and Lumsden, 1996; Le Douarin and Kalcheim, 1999). The first pharyngeal arch is subdivided along the dorsoventral axis into the maxillary and mandibular arches, which give rise to the upper and lower jaws, respectively. Recently, molecules involved in the determination of their identities have been explored and the Dlx genes, vertebrate Distal-less homologs, have been thought to play a key role (Depew et al., 2005; Merlo et al., 2000). Among the six known Dlx genes, Dlx5 and Dlx6 are expressed in the ventral part within the anterior pharyngeal arches (Depew et al., 2005; Merlo et al., 2000). Dlx5/Dlx6 double null-mutant mice demonstrate homeotic transformation of the lower jaw into an upper jaw, indicating that Dlx5 and Dlx6 are major determinants of the mandibular identity (Beverdam et al., 2002; Depew et al., 2002).

The endothelin (Edn) system, composed of three peptide ligands (Edn1, Edn2 and Edn3) and their two G-protein-coupled receptors [endothelin type A receptor (Ednra) and type B receptor (Ednrb)], is involved in diverse biological events (Kedzierski and Yanagisawa, 2001; Kurihara et al., 1999; Masaki, 2004). These receptors activate an overlapping set of G proteins (e.g. $G_q/G_{11}$), leading to various intracellular responses such as activation of phospholipase C, increase in intracellular calcium and induction of early responsive genes (Kedzierski and Yanagisawa, 2001). During embryogenesis, the Edn1-Ednra axis regulates craniofacial and cardiovascular morphogenesis, whereas the Edn3-Ednrb axis contributes to melanocyte and enteric neuron development (Kedzierski and Yanagisawa, 2001; Kurihara et al., 1999; Masaki, 2004).

In craniofacial development, Edn1 is expressed in the epithelium and mesodermal core of the pharyngeal arches, whereas Ednra is in neural crest-derived ectomesenchyme (Clouthier et al., 1998; Kurihara et al., 1995; Kurihara et al., 1994; Maemura et al., 1996). Defects in the Edn1/Ednra pathway results in the malformation of pharyngeal-arch-derived craniofacial

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structures in mice (Clouthier et al., 1998; Kurihara et al., 1995; Kurihara et al., 1994), rats (Spence et al., 1999), birds (Kempf et al., 1998) and fish (Kimmel et al., 2003; Miller et al., 2000). Homeotic transformation of the mandibular arch with downregulation of Dlx5/Dlx6 in Edn1Δ-null embryos implicates the Edn1/Ednra pathway in the dorseoventral axis patterning of the pharyngeal arch system as a positive regulator of Dlx5/Dlx6 expression (Kurihara et al., 1994; Ozeki et al., 2004; Ruest et al., 2004). Although the Gq/G11-mediated signaling pathway has been suggested to be involved in pharyngeal arch development (Dettlaff-Swiercz et al., 2005; Ivey et al., 2003; Offermanns et al., 1998), the intracellular signaling pathway coupling the Edn1/Ednra system to the induction of Dlx5/Dlx6 remains unknown.

To investigate the intracellular signaling mechanism involving the Edn1/Ednra pathway in craniofacial development, we choose a knock-in strategy with a recombinase-mediated cassette exchange (RMCE) using the Cre-lox system. This method enables an efficient exchange of a chromosomal region flanked by incompatible mutant lox sequences for a cassette located on a plasmid, and is highly advantageous in that it allows the repetitive use of the same embryonic stem (ES) cell line to insert various genes of interest into the identical recombinant allele (Sorrell and Kolb, 2005). Here we have established an Ednra knock-in system using RMCE. Knock-in of the lacZ gene resulted in the visualization of Ednra-expressing cells. Furthermore, knock-in of Ednra and Ednrb revealed Ednra-selective and non-selective signaling pathways operating in distinct regions. Ednrb knock-in mice, which demonstrated homeotic transformation of the lower jaw into an upper jaw but had relatively well-developed incisive alveolar bone and hyoid, resembled neural crest-specific Gq/G11-deficient mice. Together with differences in distal pharyngeal-arch-derived structures between these mice and Ednra-null mice, these results indicate that Gq/G11-dependent and -independent Edn1/Ednra pathways, which may correspond to Ednra-selective and non-selective signaling, respectively, are used in different contexts during pharyngeal arch development.

MATERIALS AND METHODS

Plasmid construction

A C57BL6-derived BAC clone containing the mouse Ednra gene was obtained from BACPAC Resource Center (Oakland). The targeting construct was designed to replace a 1.0 kb sequence containing the ATG translation start site in exon2 with the PGK-neomycin resistance gene cassette (Neo) flanked with lox71 at the 5’ end and lox2272 with the bovine growth hormone polyadenylation signal at the 3’ end (see Fig. 1). A PCR-amplified 1.1 kb fragment encompassing intron1 to exon2 and a 9.2 kb ClaI-BamHI fragment from intron 2 were placed on each side of the floxed Neo in a pKO Scrambler NTKV-1904 vector (Stratagene). The MC1-thymidine kinase cassette (TK) was placed 3’ downstream to the 9.2 kb fragment for negative selection.

The RMCE-mediated knock-in vector was made in a p66-2272 plasmid (Araki et al., 2002). The PGK-puromycin resistance gene cassette (Puro) was flanked by Fpl recombinase target (FRT) sequences (Schlake and Bode, 1994) and placed between lox66 and lox2272 with multiple cloning sites. For the knock-in of lacZ, the lacZ gene with an SV40 large T antigen-derived nuclear localization signal was introduced into the multi cloning site placed between lox66 and the FRT-flanked Puro using appropriate restriction enzymes. For the knock-in of Ednra and Ednrb, PCR-amplified fragments encoding the open reading frame of mouse Ednra and Ednrb cDNA were introduced into the knock-in vector in the same way.

Homologous recombination and RMCE in ES cells

The targeting vector was linearized and electroporated into B6129F1- derived ES cell line ATOM1 (Amano et al., unpublished). Clones showing positive-negative selection with neomycin and FIAU were screened for homologous recombination with genomic PCR using diagnostic primers. Correct recombination was confirmed by Southern blotting using the probes indicated in Fig. 1. For RMCE, the ES cell line in which the initial recombination was successfully achieved was used repeatedly. ES cells were infected with AxCANCre, recombinant adenovirus expressing the recombinase Cre tagged with a nuclear localization signal under the control of the CAG promoter (Kanega et al., 1995). Forty-eight hours later, ES cells were electroporated with the knock-in vector and seeded onto multidrug-resistant embryonic fibroblasts derived from the DR4 mouse strain (Tucker et al., 1997). Clones selected with 1 µg/ml puromycin were picked up and genotyped by PCR to identify RMCE-mediated recombination.

Mutant mice

Targeted ES clones were injected into ICR blastocysts to generate germline chimeras. For excision of the FRT-flanked Puro, the Fpl recombinase-expression plasmid pCAGGS-FLPe (Gene Bridges, Dresden, Germany) was injected into the male pronuclei of fertilized eggs having the knocked-in allele. Sequential recombination events were verified by PCR with specific primers, the sequences of which are available on request. Mutant mice were intercrossed with ICR mice and F2 to F5 offspring were subjected to analysis. P0-Cre-Δloxfl/fl;GaxΔlox71Δ/Δ mice were described previously (Dettlaff-Swiercz et al., 2005). All the animal experiments were performed in accordance with the guidelines of the University of Tokyo Animal Care and Use Committee.

RT-PCR

Expression of knocked-in genes was confirmed by RT-PCR. Primers p (5’-CTGATCCCGCCGACCCGGTGGA-3’) and q (5’-TACGTCGCGTATAAGTAGTCATAGCAGCAGTA-3’) were designed to detect transcripts from both Ednra and Ednrb knocked-in alleles as a 284 bp band. The combination of primers p and r (5’-TCAATGACCAGTGAATAAGGT-3’) was designed to detect both endogenous and knocked-in Ednra transcripts as 753- and 659 bp bands, respectively. Primer s (5’-GAGAATGACGCGTGGCTT-3’), with primer p, was designed to detect specifically knocked-in Ednrb transcripts as a 477 bp product. The housekeeping gene GAPDH served as internal control.

Skeletal staining

Alizarin Red/Alician Blue staining was performed, as previously described (McLeod, 1980).

β-Galactosidase staining

lacZ expression was detected by staining with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) for β-galactosidase activity. Whole-mount staining was performed as previously described (Nagy et al., 2003) with minor modifications. For sections, samples were embedded in OCT compound, cryosectioned and subjected to X-gal staining. Some sections were counterstained with 1% Orange G (Sigma).

In situ hybridization

Whole-mount in situ hybridization was performed as described previously (Wilkinson, 1992). Probes for Hand2 (Srivastava et al., 1995) and goosecoid (Yamada et al., 1995) were generously provided by D. Srivastava (University of California, San Francisco, CA) and G. Yamada (Kumamoto University, Kumamoto, Japan), respectively. Other probes were prepared by RT-PCR as described (Ozeki et al., 2004).

Apoptosis analysis

For detection of apoptotic cells, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed on 14 µm consecutive frozen sections of embryonic day 10.5 (E10.5) embryos using a DeadEnd Labeling kit (Promega) with diaminobenzidine as substrate, as previously described (Abe et al., 2007).

RESULTS

Generation of Ednra-lacZ knock-in mice by RMCE

We first introduced an exchangeable floxed site into the Ednra locus in ES cells to generate the Ednra<sup>neo</sup> allele, which could serve as a target for RMCE (Fig. 1). Electroporation of 1 × 10<sup>7</sup> ES cells with
**Fig. 1. RMCE in the mouse Ednra gene.** (A) Strategy for targeting and knock-in of lacZ. Probes for genotyping are indicated as 5'- and 3'-probes. B, BamHI; Bl, BlnI; C, ClaI; P, PstI; Pm, Pnec. (B) Southern blot analysis of PstI- or BlnI/Pnec-digested genomic DNA of the offspring from an intercross of heterozygotes, probed with the 5'- and 3'-probes, respectively. (C) Genomic PCR analysis for sequential Cre-lox-mediated recombination with primers indicated in A.

The targeting vector followed by positive-negative selection yielded 224 clones surviving selection, among which 13 clones proved to be correctly recombinatd and five clones were transmitted through the germline.

One recombinant ES clone capable of efficient germline transmission was used repeatedly for subsequent RMCE. In the present scheme, the expression of a knocked-in gene was expected to recapitulate the endogenous Ednra expression patterns. To confirm this and to evaluate the efficiency of RMCE, we introduced the lacZ gene into the Ednra locus (Ednra<sup>lacZ2FRT</sup>). After infection of AxCANCre and electroproetation of nls-lacZ-containing knock-in vector, positive selection with puromycin yielded 94 surviving clones, among which 82 clones (87%) were correctly introduced with lacZ. Three lacZ-carrying clones were transmitted to the germline. Subsequently, we microinjected pCAGGS-FLPe into fertilized eggs obtained from ICR females crossed with Ednra<sup>lacZ2FRT+</sup> males to obtain Ednra<sup>lacZ2FRT+/+</sup> mice in which the FRT-flanked Puro was removed. All the heterozygotes were viable and fertile.

**lacZ-labeling of Ednra-expressing cells during embryonic development**

To analyze the expression patterns of Ednra during postimplantation development, we performed β-galactosidase staining on Ednra-lacZ knock-in embryos. lacZ expression patterns were identical between Ednra<sup>lacZ2FRT+</sup> and Ednra<sup>lacZ2FRT+/+</sup> embryos, indicating that the presence of Puro in the mutant allele did not significantly affect the expression of knocked-in genes. Thereafter, pictures of Ednra<sup>lacZ2FRT+</sup> embryos stained for β-galactosidase activity are shown.

At E8.25 to 8.5, lacZ expression was observed in the head mesenchyme at the hindbrain level (Fig. 2A-C). High magnification of transverse sections at E8.5 revealed lacZ expression in migratory neural crest cells delaminating from the dorsal neuroepithelium (Fig. 2D). By contrast, lacZ expression was undetectable in the neuroepithelium, including the premigratory neural crest, surface ectoderm, foregut endoderm and vascular endothelium (Fig. 2C,D). At E9.0, lacZ was highly expressed in the head and pharyngeal arch regions, in the heart, and in the ventral half of the trunk (Fig. 2E). In the first to third arches, lacZ expression was detected in neural-crest-derived ectomesenchymal cells, whereas the pharyngeal ectoderm and endoderm, vascular endothelium and many cells in the core mesenchyme were lacking lacZ expression (Fig. 2F). lacZ expression was also extensively observed in the head mesenchyme adjacent to the neuroepithelium (Fig. 2F). In the cardiac outflow region, cardiac neural crest cells surrounding the second and third pharyngeal arch arteries and colonizing between the foregut and the aortic sac showed intense lacZ expression (Fig. 2G). These lacZ expression patterns appeared to recapitulate endogenous Ednra expression revealed by in situ hybridization in whole mounts (Fig. 2H) and in sections (Clouthier et al., 1998; Yanagisawa et al., 1998).

By comparison, in situ hybridization of E9.0 embryos for Crabp1, a neural crest marker (Ruberte et al., 1992), revealed its expression in the streams of migratory neural crest cells, which overlapped with Ednra-lacZ expression (Fig. 2I). However, Ednra-lacZ expression was also found in Crabp1-negative mesenchymal regions ventromedial to the neural crest streams (Fig. 2K). The expression pattern of Snail1, which is expressed in neural-crest- and mesoderm-derived head mesenchyme before E10.5 (Nieto et al., 1992; Smith et al., 1993), was more similar to that of Ednra-lacZ (Fig. 2J). These results suggest that lacZ-expressing mesenchymal cells are likely to originate from both neural crest and mesoderm.

At E10.0, lacZ expression was observed throughout the head mesenchyme adjacent to the neural tube (Fig. 3A,B). Trigeminal ganglia, which originate in neural crest cells and placode cells, were highly populated with lacZ-positive cells (Fig. 3B). In the pharyngeal arches, lacZ expression was mainly located in ectomesenchyme underlying the epithelium (Fig. 3A). The distribution of lacZ expression within the wall of the aortic sac and pharyngeal arch arteries corresponded to cardiac neural crest cell population (Fig. 3C). At E12.5, lacZ expression was found in ectomesenchyme underlying the oral epithelium in the lower and upper jaws (Fig. 3D,G). In developing tooth buds, lacZ expression was present in mesenchyme surrounding the endoderm-derived dental lamina (Fig. 3E). In the precartilage primordium of Meckel’s cartilage, lacZ expression was undetectable in the bilateral rod portion (Fig. 3F), but was intensely present in the rostral process (Fig. 3G,H).

**Craniofacial defects of Ednra-null mice**

As expected, Ednra<sup>neo/neo</sup>, Ednra<sup>neo/lacZ</sup> and Ednra<sup>neo/lacZ</sup> mice demonstrated perinatal lethality and craniofacial abnormalities, which were almost identical to the phenotype of mice lacking Edn1.
or Ednra (Ozeki et al., 2004; Ruest et al., 2004). In these mutants, the lower jaw appeared as a mirror image of the upper jaw with arrays of vibrissae (Fig. 4A,B). Skeletal analysis revealed transformation of mandibular arch-derived structures into maxillary arch-derived elements in mutants (Fig. 4C-F). Most of the dentary was replaced with an ectopic bone, which appeared as a mirror image duplication of the ipsilateral maxilla (Fig. 4G,H), the morphology of which was almost identical to that of wild-type maxillae (Fig. 4I). In addition, a second set of jugal and palatine bones was observed in mutants (Fig. 4E,F). In the most distal portion, alveolar bone was formed around a tiny rostral process of Meckel’s cartilage and surrounded a lower incisor, although its growth was variable among individuals (Fig. 4F,G). Zygomatic processes of the squamosal were malformed and often absent (Fig. 4F). In addition, ectotympanic and gonial bones were lost, whereas the pterygoid process, ala temporalis and lamina obturans were duplicated in mutants (Fig. 4J,K). The malleus and incus were malformed, giving an ectopic structure extending to the squamosal (Fig. 4L,M).

Among the second-arch-derived structures, the mutant hyoid was aberrantly ossified and fused bilaterally to the basisphenoid in Ednra-null mutants (Fig. 4N,O). The stapes in mutants retained its normal appearance, but was displaced out of the fenestra ovalis and was connected to the hyoid through a cartilaginous strut (Fig. 4L,M). This strut was likely to be a transformed lesser horn of the hyoid, which was fused to the hyoid body and stretched to the lateral side. In addition, the styloid process was truncated in mutants (Fig. 4L,M).

Knock-in of Ednra cDNA rescued the Ednra-null phenotype
Recapitulation of Ednra expression by the RMCE-mediated knock-in of lacZ may justify the application of this system for the functional analysis of the Edn1/Ednra signaling mechanism in embryonic development. To confirm this, we introduced Ednra cDNA into the Ednraneo allele, expecting rescue of the Ednra-null phenotype by restoring Ednra expression (Fig. 5A). The efficiency of RMCE-mediated knock-in in ES cells was 44% (41 in 94 surviving clones). Four clones generated germline chimeras.
following blastocyst injection. Chimeric and heterozygous mice carrying the Ednra knock-in allele (Ednra<sup>lacZ<sup>+</sup></sup>) were intercrossed with Ednra<sup>lacZ<sup>+/+</sup></sup> mice to obtain Ednra<sup>lacZ<sup>+/−</sup></sup> offspring.

Mice with the genotype Ednra<sup>lacZ<sup>+/−</sup></sup> were viable and fertile. Their appearance (Fig. 5B) and craniofacial skeletal morphology (Fig. 5D) were indistinguishable from those of wild-type and Ednra<sup>lacZ<sup>+/+</sup></sup> heterozygous mice (Fig. 4A,C). These results indicate that the knocked-in Ednra cDNA can restore the function of endogenous Ednra at least in craniofacial development, which validates the usefulness of the present RMCE-mediated knock-in system for functional analysis.

**Knock-in of Ednr<sup>b</sup> cDNA could only partially rescue the Ednra-null phenotype**

Previous studies have demonstrated that Edn1 can bind to both Ednra and Ednr<sup>b</sup> receptors with similar affinities and activate common signaling pathways, including G<sub>q</sub>/G<sub>11</sub>-dependent signals in many cells (Cramer et al., 2001; Jouneaux et al., 1994; Kedzierski and Yanagisawa, 2001; Masaki et al., 1999; Takigawa et al., 1995).

If Ednra and Ednr<sup>b</sup> are interchangeable in the context of pharyngeal arch development, Ednr<sup>b</sup> knock-in is expected to rescue the Ednra-null phenotype. To test this possibility, we introduced Ednr<sup>b</sup> cDNA into the Ednra<sup>deo</sup> allele in the same procedure as Ednra knock-in (Fig. 5A). Among 12 knock-in clones obtained through the screening, two clones were used to generate germline chimeras. Resultant heterozygous mice with the Ednr<sup>b</sup>-knock-in allele (Ednra<sup>deo</sup>) were intercrossed with Ednra<sup>deo</sup> or Ednra<sup>lacZ<sup>+</sup></sup> mice. The expression levels of Ednr<sup>b</sup> from the single knock-in allele were comparable to those of Ednra as estimated by RT-PCR analysis of E9.5 mandibular arches (Fig. 5A).

Unexpectedly, all the Ednra<sup>deo</sup> and Ednra<sup>lacZ<sup>+</sup></sup> mice died at birth and demonstrated craniofacial abnormalities similar to Ednra-null mice. In E18.5 Ednra<sup>lacZ<sup>+/−</sup></sup> mice, the appearance of the lower jaw was a mirror image of the upper jaw with vibrissae (Fig. 5C). Skeletal analysis of Ednra<sup>deo</sup>-knock-in mice demonstrated a duplication of the upper jaw elements in the lower jaw region (Fig. 5E). Similar abnormalities were manifested in Ednra<sup>B</sup>-null mice, in which both of the two Ednra alleles were replaced with knocked-in Ednr<sup>b</sup> cDNA (data not shown).

The skeletal phenotype of Ednra<sup>deo</sup> and Ednra<sup>B</sup> mice, however, was different from that of Ednra-null mice in some respects. First, whereas the distal portion of the Ednra-null mandible was variable and often severely hypoplastic, failing to fuse at the midline (Fig. 4F,G), all the Ednra<sup>deo</sup> (n=7) and Ednra<sup>B</sup> (n=9) mice examined had relatively well-developed incisive alveolus, the appearance of which was similar to that of the normal dentary (Fig. 5E,F). Second, the hyoid in some Ednr<sup>b</sup>-knock-in mice was fused unilaterally to the basisphenoid (in one of seven Ednra<sup>deo</sup> mice and four of nine Ednra<sup>B</sup> mice) or separated from the basisphenoid (in three of nine Ednra<sup>B</sup> mice) although it typically had an extended ossification center and was different from that of Ednra-null mice, in some respects. First, whereas the distal portion of the Ednra-null mandible was variable and often severely hypoplastic, failing to fuse at the midline (Fig. 4F,G), all the Ednra<sup>deo</sup> (n=7) and Ednra<sup>B</sup> (n=9) mice examined had relatively well-developed incisive alveolus, the appearance of which was similar to that of the normal dentary (Fig. 5E,F). Second, the hyoid in some Ednr<sup>b</sup>-knock-in mice was fused unilaterally to the basisphenoid (in one of seven Ednra<sup>deo</sup> mice and four of nine Ednra<sup>B</sup> mice) or separated from the basisphenoid (in three of nine Ednra<sup>B</sup> mice), whereas all the Ednra<sup>deo</sup> mice compared (n=10) showed bilateral hyoid-basisphenoid fusion. The hyoid in Ednra<sup>deo</sup> and Ednra<sup>B</sup> mice, whether it was fused to or separated from the basisphenoid, had nearly normal appearance compared with that of Ednra-null mice, although it typically had an extended ossification center and connected to the stapes though a transformed lesser horn as in Ednra<sup>deo</sup> mice (Fig. 5G). Thirdly, ectopic cartilage appeared between the malleal/incal region and the extended basitrabecular process and/or ala temporalis in four of seven Ednra<sup>deo</sup> mice and six of nine Ednra<sup>B</sup> mice (Fig. 5H), whereas similar cartilage was observed in none of ten Ednra<sup>deo</sup> mice (Fig. 4L). The first two differences may indicate that Ednr<sup>b</sup> could partially rescue the Ednra-null phenotype in the distal portion of the pharyngeal arch structures.

It has recently been reported that apoptotic cells are increased and extend distally in the mandibular arch mesenchyme of E9.5-10.5 Ednra-null embryos (Abe et al., 2007). To test whether knocked-in Ednr<sup>b</sup> could prevent increased apoptosis, we performed TUNEL staining on sections of E10.5 embryos. Consistently with the previous

![Fig. 3. LacZ expression in the head mesenchyme and neural-crest derivatives in Ednra<sup>deo</sup> mouse embryos. (A-C) Transverse sections at E10.0. LacZ expression is observed throughout the head mesenchyme adjacent to the neural tube and in the peripheral pharyngeal arch mesenchyme (A). Trigeminal ganglia are highly populated with lacZ-positive cells (B). LacZ expression is detectable within the lateral wall of the aortic sac and pharyngeal arch arteries (C). (D-H) Transverse sections at the levels of the central (D-F) and distal (G,H) regions of the lower jaw at E12.5. E,F,H are high magnification images of the boxed areas in D and G. LacZ expression is observed in mesenchyme underlying the oral epithelium in the lower and upper jaws (D,G). In the molar (E) and incisor (H) buds, lacZ expression is present in mesenchyme surrounding the dental lamina. LacZ expression is undetectable in the precartilage primordium contributing to the rod portion of Meckel's cartilage, whereas surrounding mesenchymal cells express lacZ (F). In the primordium of the rostral process of Meckel's cartilage, lacZ is highly expressed (H). Scale bars: 100 μm in A-C; 500 μm in D,G; 50 μm in E,F,H. aa2, second arch artery; da, dorsal aorta; dl, dental lamina; dm, dental mesenchyme; fg, foregut; gV, trigeminal ganglion; lj, lower jaw; nt, neural tube; pa1/2, first and second pharyngeal arches; pc, precartilage primordium of Meckel's cartilage; to, tongue; uj, upper jaw.](image-url)
report, TUNEL-positive cells extended from the proximal to the distal mandibular region in E10.5 Ednra<sup>neo/neo</sup> embryos (n=5), whereas TUNEL-positive cells were confined to the proximal region in wild-type embryos (n=6) (Fig. 6A,B). Similarly, Ednra<sup>B</sup> embryos also showed apparently increased numbers of TUNEL-positive cells extending into the distal mandibular region (n=4) (Fig. 6C).

We further examined gene expression profiles in Ednrb-knock-in embryos by whole-mount in situ hybridization. LacZ signals were equally distributed within the pharyngeal arch region in E9.5 control, Ednra<sup>lacZ/B</sup> (Fig. 7A,B) and Ednra<sup>-null</sup> (data not shown) embryos, suggesting that Ednra-positive cells were present there to a similar extent. In the pharyngeal arch mesenchyme of E9.5 Ednra<sup>B/B</sup> embryos, the expression of Dlx3, Dlx5 and Dlx6 is downregulated (Fig. 7C-H), as in Edn1<sup>-/-</sup> and Ednra<sup>-/-</sup> embryos (Charite et al., 2001; Clouthier et al., 2000; Ozeki et al., 2004). The expression of Hand2 and Goosecoid was also downregulated in Ednra<sup>-/B</sup> pharyngeal arches (Fig. 7I-L), as in Edn1<sup>-/-</sup> and Ednra<sup>-/-</sup> embryos (Clouthier et al., 1998; Thomas et al., 1998) as well as in Dlx5<sup>-/-</sup>:Dlx6<sup>-/-</sup> embryos (Charite et al., 2001; Depew et al., 1999; Depew et al., 2002). Taken together with morphological and apoptosis analysis, these results indicate that Ednrb cannot substitute for Ednra in pharyngeal development, although it may partially rescue the Ednra-null phenotype.

**Comparison of craniofacial structures between Ednra-modified mice and neural-crest-specific G<sub>A01</sub>G<sub>A01</sub>-deficient mice**

Offermanns’s group has demonstrated that P0-Cre<sup>+-x</sup>G<sub>fl<sup>lox</sub>/lox</sub>G<sub>A01</sub>-<sub>A01</sub> mice lacking G<sub>A01</sub>G<sub>A01</sub> in a neural-crest-specific manner show craniofacial defects similar to those in Edn1<sup>-/</sup>- or Ednra<sup>-/-</sup> deficient mice (Dettlaflf-Swiercz et al., 2005). However,
FIG. 5. Phenotypes of Ednra and Ednrb knock-in mice. (A) RMCE-mediated knock-in of Ednra and Ednrb cDNA into the Ednra locus. Partial structures of the wild-type and knocked-in Ednra allele and RT-PCR analysis for the expression of knocked-in genes in mandibular arches of E9.5 Ednra- and Ednrb-knock-in heterozygous embryos are shown. PCR primers were represented by arrowheads in the left panel. Note that 753- and 659-bp bands correspond to knocked-in and endogenous Ednra transcripts, respectively. (B-H) Facial appearances (B,C) and skeletal structures (D-H) of E18.5 EdnraalacZ/A (Ednra-knock-in) (B,D) and EdnraalacZ/B (Ednrb-knock-in) (C,E-H) mice. Ednra knock-in restored normal facial appearance (B) and skeletal structures (D). Ednrb knock-in mice still exhibit craniofacial defects resembling the Ednra-null phenotype (C,E), except for some differences described below. White arrowhead, ectopic cartilage connecting the incus and extended basitrabecular process. (f) Comparison of the morphology of incisive alveolar bones (boxed with dotted line) among the Ednra-null (EdnralacZ/B), Ednrb-knock-in (EdnraalacZ/B) and wild-type (Ednra+/+) mandible. Incisors were removed. The EdnraalacZ/B incisive alveolus is well developed, comparable with the wild-type one. (G) A representative of the EdnraalacZ/B hyoid, which is, unlike the Ednra-null hyoid, not fused to the basisphenoid. The body has an extended ossification center and is connected to the stapes through a transformed lesser horn. The great horn is fused with the superior horn of the thyroid (arrow). (H) Ectopic cartilage (arrowhead) between the malleal/incal region and ala temporalis in EdnraalacZ/A mice. at, ala temporalis; bh, body of hyoid; bs, basisphenoid; dnt, dentary; fovl, fenestra ovalis; ghh, greater horn of hyoid; hy, hyoid; in, incus; jg, jugal; lh, lesser horn of hyoid; li, lower incisor; ma, malleus; mx, maxilla; pmx, premaxilla; sp, styloid process; st, stapes; zpmx, zygomatic process of maxilla; *, ectopic structure.

**DISCUSSION**

Previous studies have implicated Edn1/Ednra signaling as a mediator of regional specification and morphogenesis in craniofacial development in vertebrates. To further investigate the intracellular mechanism underlying the involvement of the Edn1/Ednra signaling in embryonic development, we adopted RMCE using the Cre-lox system to introduce genes of interest into the Ednra locus in mouse ES cells systematically and efficiently. Indeed, the efficiency of RMCE-mediated knock-in recombination was very high in our screening (44 to 87%), compared with conventional homologous recombination. This is similar to or higher than the efficiencies previously reported for RMCE using the Cre-lox system (Araki et al., 2006; Liu et al., 2006; Toledo et al., 2006) or the Flp-FRT system (Cesari et al., 2004; Roebroek et al., 2006). In our RMCE procedure, we replaced a 1.0 kb genomic sequence with knock-in sequences containing mutant lox sites and an FRT-flanked Puro. The expression of knocked-in lacZ appeared to recapitulate endogenous

detailed description in terms of this similarity has not yet been reported. To clarify the relationship between Edn receptors and Gq/11 signaling in neural-crest-derived ectomesenchyme, we revisited the craniofacial phenotype of neural-crest-specific Gq/11-deficient mice.

In addition to the duplication of the jugal and maxillary bones in the mandibular region, Gq/11-deficient mice had duplicated palatine, pterygoid and lamina obturans (Fig. 8A-D), as observed in Ednra-null (Fig. 4D,F) and Ednrb-knock-in (Fig. 5E, Fig. 8E) mice. Unlike Ednra-null mice, however, the incisive alveolar bone in the distal mandibular region was relatively well developed in all the Gq/11-deficient mice examined (n=8) (Fig. 8B,D,F). This morphological feature is reminiscent of the Ednrb-knock-in phenotype (Fig. 8E,G) rather than the Ednra-null one (Fig. 8H). The basitrabecular process was abnormally extended to the ear region in all the cases (Fig. 8B,D). Furthermore, the hyoid of Gq/11-deficient mice was not fused to the basisphenoid in all the cases (Fig. 8I,J). Instead, the body has an extended ossification center and is fused with the lesser horn and often with the superior horn of the thyroid (Fig. 8I,J), as seen in Ednrb-knock-in mice (Fig. 5G). These similarities suggest that Gq/11 mainly mediate Ednra-selective signaling in neural-crest-derived mesenchyme during pharyngeal arch development, but the Ednrb-replaceable signaling is likely to be mediated by the Gq/11-independent pathway.
Ednra expression irrespective of the presence of Puro, and the knock-in of Ednra cDNA could normalize craniofacial defects caused by Ednra-null mutation. Furthermore, no additional abnormalities were evident in any knock-in mice after RMCE procedure at least in terms of embryonic development, viability and fertility. These results support the relevance of our RMCE-mediated knock-in procedure for analysis of gene function in craniofacial development.

**Ednra-lacZ expression in the head mesenchyme and cranial neural crest derivatives**

Previous studies have indicated that Edn1 acts on cranial/cardiac neural crest cells through Ednra and activates transcriptional machinery responsible for doroventral patterning (Charite et al., 2001; Ozeki et al., 2004; Ruest et al., 2004). The crucial window for this Edn1-Ednra interaction in pharyngeal arch development is around E8.5 to 9.0, when regional identities start to be specified within the anterior pharyngeal arches (Fukuhara et al., 2004). Consistently, Ednra-lacZ expression was detectable just before this stage (E8.25; ~6-somite stage) in the head mesenchyme, including migratory neural crest cells delaminating from the dorsal neuroepithelium. By contrast, premigratory neural crest cells in the neural plate did not express lacZ, suggesting that the induction of Ednra expression may be coupled with epithelial-mesenchymal transition. Thereafter, Ednra-expressing neural crest cells migrating into the ventral region of the anterior pharyngeal arches are supposed to receive the Edn1 signal, leading to the upregulation of Dlx3/Dlx6 expression and the specification of a ventral identity.

In addition to the expression in neural-crest-derived mesenchymal cells, Ednra-lacZ was likely to be expressed in the mesoderm-derived head mesenchyme. This is further supported by the difference in the pattern of lacZ expression between the Ednra-lacZ mice and other mice in which neural crest cells are specifically marked. Protein 0 (P0)-Cre transgenic mice harboring a conditional lacZ allele, for example, demonstrated lacZ expression broadly in neural-crest derivatives (Yamauchi et al., 1999). The Wnt1-Cre transgene also directed the expression of a conditional lacZ allele specifically to neural crest cells in mice (Chai et al., 2000). In both cases, the head mesenchyme adjacent to the neural tube was largely lacZ-negative. In fact, the head mesenchyme originated from both the cranial paraxial mesoderm and neural crest (Trainor and Tam, 1995). Thus, Ednra appears to be extensively expressed in mesoderm-derived mesenchyme (except for the pharyngeal core mesoderm and vascular endothelium) as well as in neural-crest-derived ectomesenchyme in the craniofacial region.

At later stages, Ednra-lacZ expression was observed in many cranial/cardiac neural crest derivatives. In the Meckel’s cartilage primordium, which is also derived from neural crest cells, lacZ expression was detected only in the rostral process, a distalmost portion, at E12.5. At E12.5 to 13.5, the rostral process is rich in proliferative, undifferentiated cells, while cells start to differentiate into chondrocytes in the bilateral rod portion (Ramaesh and Bard, 2003). Thus, Ednra expression in neural-crest derivatives may be stage- and/or lineage-dependent.

**Diversity of Edn receptor signaling in pharyngeal arch development**

Ednra and Ednrb share common ligand affinities and downstream signaling pathways. In particular, the Gq/G11-mediated pathway, which is assumed to be responsible for Edn1/Ednra-dependent craniofacial development, is also activated by Ednrb stimulation in various cell types (Cramer et al., 2001; Jouneau et al., 1994; Masaki et al., 1999; Takigawa et al., 1995). However, knock-in of Ednrb failed to rescue homeotic transformation of the lower jaw into
Ednrb function in the specification of mandibular identity in knock-in mice. This result indicates that Ednrb cannot restore Ednra function in the development of the distal mandibular region (arrowheads), as Ednra–/– mice. Unlike Ednra-null mice, the Gq/G11-deficient hyoid is not fused to the basisphenoid. Instead, the body has an extended ossification center and is fused with the lesser horn of the hyoid and the superior horn of the thyroid (arrow). bh, body of hyoid; bs, basisphenoid; btp, basitrabecular process; dnt, dentary; etm, ectotympanic; ghh, greater horn of hyoid; hy, hyoid; ina, incisive alveolus of dentary; jg, jugal; lhh, lesser horn of hyoid; lo, lamina obstructor; mx, maxilla; pl, palatine; ptx, premaxilla; ptg, pterygoid; sht, superior horn of the thyroid; sq, squamosal; *, ectopic structure.

An upper jaw-like structure in Ednra-null mice, although knocked-in Ednrb appeared to be expressed at levels similar to those of knocked-in Ednra. This result indicates that Ednrb cannot restore Ednra function in the specification of mandibular identity in pharyngeal arch development.

One possible explanation for the failure of rescue by Ednrb knock-in is that Ednrb might not activate Gq/G11 adequately enough to elicit downstream signals necessary for the ventral specification of the pharyngeal arches. Indeed, Ednrb is less potent in Gq coupling in some cells and in reconstituted phospholipid vesicles (Doi et al., 1999; Takigawa et al., 1995). It is also possible that Ednrb may not associate with Gq/G11 in cranial neural crest cells in a physiological context. In trunk neural crest development giving rise to the enteric nerve system, the Edn3/Ednrb signal is likely to be mediated by inhibition of protein kinase A (Barlow et al., 2003), which appears to be independent of Gq/G11. Thus, Ednra and Ednrb may couple to different G proteins, leading to activation of distinct signaling pathways. Another possibility is that difference in receptor desensitization or differences in the potency and/or efficacy of the receptor agonists with regard to receptor activation might cause the failure of rescue by Ednrb. Further studies are required to test these possibilities.

By contrast, there were some differences in craniofacial defects between Ednra-null and Ednrb knock-in mice. Unlike Ednra-null mice, Ednrb knock-in mice had a relatively well-developed incisive alveolar bone of the mandible and, in some cases, the hyoid separated from the basisphenoid as in normal mice. Extended basitrabecular process with connection to ectopic cartilage was often observed in Ednrb knock-in mice. The first two differences suggest that Ednrb may partially replace Ednra function in the development of distal structures in the mandibular and hyoid arches. Considering the regional heterogeneity in Ednra expression within the Meckel’s primordium, the Edn1/Ednra signal may be also required for the formation of the distal region later than the stage of dorsoventral specification. Interestingly, craniofacial defects of neural-crest-specific Gq/G11-deficient mice were very similar to those of Ednrb knock-in mice rather than Ednra-null mice. This similarity suggests that aspects of the Edn1/Ednra signaling that Ednrb can substitute may be mediated by a Gq/G11-independent pathway. Thus, Edn1/Ednra may activate different G proteins in different contexts during pharyngeal arch development. Alternatively, Edn receptor functions in cells other than the neural-crest derivatives may also be required for pharyngeal arch development. The mechanism underlying the selectivity of the Edn receptors in terms of G-protein coupling in cranial neural crest cells in different contexts should be clarified in further investigations.
Regionality within the mandible in terms of the requirement for Edn1/Ednra-Gq/G11 signaling

The present study showed that the proximal portion of the dentary underwent homeotic transformation into upper jaw elements, whereas the distal portion maintained its mandibular identity in Ednra-null, Ednrb-knock-in and Gq/Gq11-null mice. Notably, the ectopic bone replacing the dentary in mutant mice resembled the maxilla, leaving incisive alveolar bone with mandibular identity in defects in this pathway, although apoptosis in Gq/Gq11-null mice has not yet been analyzed. By contrast, this signaling pathway involved in this pathway. The possible dual roles of the Edn1/Ednra signaling in pharyngeal arch development are summarized in Fig. 9.

The incisive alveolus appears to be equivalent to the premaxilla in the upper jaw, as extrapolated from the conjugation to the maxillary-like bone in Ednra-null mutants (Fig. 4G,H). This portion and the premaxilla are missing in Dlx5/6−/− mice, indicating that these structures are dependent on Dlx5 and Dlx6 (Beverdam et al., 2002; Depew et al., 2002). Residual Dlx5 and Dlx6, independent of the Edn1/Ednra to Gq/G11 signaling, may be responsible for the formation of these distal structures.

Usefulness of the present RMCE-mediated knock-in system in studies on craniofacial development

The present RMCE-mediated knock-in procedure enabled us to introduce a series of gene cassettes to be expressed in a spatiotemporal pattern similar to the endogenous Ednra. The efficiency was much higher than that of conventional homologous recombination in ES cells. Using this procedure, we could examine the expression pattern and function of Ednra in craniofacial development. In particular, differences in Ednra and Ednrb knock-in have provided a clue to further analysis of the receptor domain function. Furthermore, this system can be broadly applicable for studies on gene function, including cell fate determination, differentiation, regional specification and so on. These applications will largely contribute to our understanding of the molecular mechanisms that regulate craniofacial development.

References


segmentation is preserved throughout craniofacial ontogeny. Development 122, 3239-3242.


