論文の内容の要旨

論文題目：

Mechanisms Regulating the Development of Thoracic Great Vessels by the Endothelin Signaling

（エンドセリンシグナルによる胸部大血管発生調節のメカニズム）

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Backgrounds

In mammals, pharyngeal arch arteries are initially formed as symmetric pairs of arteries that connect aortic sac with dorsal aortae. They undergo a remodeling process and finally form thoracic arteries including thoracic aorta, pulmonary artery and ductus arteriosus. The abnormal remodeling process of pharyngeal arch arteries in human results in congenital vascular anomalies such as hypoplastic or interrupted aortic arch.

Cranial neural crest cells are known to be committed to the remodeling process of pharyngeal arch arteries. During neural tube closure, they migrate in distinct streams and contribute to head mesenchyme together with mesodermal cells. Cardiac neural crest cells, constituting a subpopulation of cranial neural crest cells, migrate to the 3rd, 4th and 6th pharyngeal arch arteries, aortic sac, and the conotruncal region of heart, and contribute to the medial layer of great arteries and aortopulmonary septum.

Endothelin-1 (Edn1) was originally identified as a vascular endothelium-derived vasoconstrictor. Edn1 binds to its G-protein coupled receptor, Endothelin receptor type A (Ednra), and contributes to the development of pharyngeal arches. Defects in the Edn1/Ednra pathway result in the malformation of pharyngeal-arch-derived craniofacial structures and thoracic arteries in mice.

Homeotic transformation of the lower jaw into the upper jaw structure with downregulation of the homeobox genes *Dlx5/Dlx6* in *Edn1*-null embryos indicate the involvement of the Edn1/Ednra pathway in the dorsoventral axis patterning of pharyngeal arch system as a positive regulator of *Dlx5/Dlx6* expression.

In contrast to the Edn1/Ednra-dependent pathway involved in craniofacial patterning, the pathway involved in pharyngeal arch artery remodeling to form thoracic arteries is largely unknown. The aim of this study is to clarify the mechanism by which the Edn1/Ednra signaling regulates pharyngeal arch artery remodeling.

Methods

Ednra$^{+/lacZ}$, *Ednra*$^{+/EGFP}$ (*lacZ* or *EGFP* knock-in) mice and *Edn1*$^{-/-}$ mice have been described previously. By crossing these mice, the *Ednra*$^{lacZ/EGFP}$ (*Ednra*-null) mice were obtained. *Ednra*$^{Ednrb/Ednrb}$ (*Ednrb* knock-in) mice have been also reported, in which not *Ednra* but *Ednrb* is expressed under the control of the *Ednra* gene promoter. *Dlx5/Dlx6* double knock-out mice have been reported previously. *Wnt1-Cre* mice, in which Cre recombinase is expressed in early neural crest cells, were crossed with R26R reporter mice containing a *loxP*-flanked *lacZ* cassette in the *Rosa26* locus, to generate *Wnt1-Cre*;*R26R* mice, in which β-galactosidase expression is observed along the neural tube and in most neural crest-derived cells. We further generated *Wnt1-Cre;R26R;Ednra*$^{EGFP/EGFP}$(*Ednra*-null) mice by crossing above-mentioned mice.

Using these mice, I performed phenotypic evaluation by ink injection, wholemount or section immunostaining for several markers, and β–galactosidase stainig.

Results

1. Edn1/Ednra signaling regulates the remodeling of thoracic arteries.

First, the phenotype of thoracic arteries in E18.5 embryos was examined by ink injection. In wild-type embryos, like in humans, three vessels branch from aortic arch; brachiocephalic, left common carotid, and left subclavian arteries. The brachiocephalic artery then bifurcates to form right subclavian and right common carotid arteries. By contrast, in *Edn1*- or *Ednra*-null embryos, abnormal vessels from bilateral common carotid arteries and abnormal bifurcation of the brachiocephalic artery were observed with high incidence, as previously described. Some *Edn1*-null embryos demonstrated more severe phenotype like type-B aortic arch interruption or hypoplastic aortic arch. These aortic arch anomalies were not observed in *Ednra*-null embryos of our ICR background, in contrast to a previous report on C57BL/6 background.

Next, the thoracic arteries of *Ednra*$^{Ednrb/Ednrb}$ (*Ednrb*-knock-in) embryos were examined. *Ednrb*-knock-in embryos demonstrated vascular anomalies similar to those of *Ednra*-null embryos, indicating that *Ednrb* gene expression cannot rescue the thoracic artery anomalies of *Ednra*-null mice. Thus, Edn1/Ednra signaling regulates the morphogenesis of thoracic great vessels, like in craniofacial development.

2. Regulation of vascular morphogenesis by the Edn-1/Ednra signaling is independent of Dlx5/Dlx6-mediated regional identification of pharyngeal arches.

In craniofacial development, the Edn1/Ednra signaling regulates the expression of the homeobox genes *Dlx5* and *Dlx6*. Deletion of *Edn1* or *Ednra* results in downregulation of *Dlx5/Dlx6* in mandibular arch and induces homeotic transformation of the lower jaw into the upper jaw structure. To investigate whether this *Dlx5/Dlx6*-mediated pathway is involved in vascular morphogenesis, the phenotype of thoracic great vessels in E18.5 *Dlx5/Dlx6* double knock-out embryos was examined. Almost all *Dlx5/Dlx6* double knock-out embryos demonstrated the normal pattern of great vessels, although they presented the formerly-reported craniofacial anomalies, the homeotic transformation of the lower jaw into the upper

jaw structure. These data indicate that, unlike craniofacial development, vascular morphogenesis does not depend on the *Dlx5/Dlx6*-dependent ventral identification of pharyngeal arches, and requires an alternative *Dlx5/Dlx6*-independent molecular pathway downstream of the Edn1/Ednra signaling.

3. The abnormal vessels from common carotid arteries derive from the abnormally persistent first and second pharyngeal arch arteries.

I focused on the abnormal vessels from the common carotid arteries in *Ednra*-null mice and sought for their origin. First, I performed the CD31 (vascular endothelial marker) immunostaining of the pharyngeal arch arteries at the forming stage (E9.5-10.5). The first and second pharyngeal arch arteries equally formed both in *Ednra*-null and heterozygous embryos at E9.5, but the regression of these arteries was less eminent in *Ednra*-null embryos at E10.5. Next, the pharyngeal arch arteries at the remodeling stage (E10.5-E12.5) were visualized by ink injection. In heterozygous embryos five pairs of symmetrical pharyngeal arch arteries underwent a remodeling process to form the final pattern of thoracic great vessels at E12.5. In this process, the first and second pharyngeal arch arteries regressed to a large extent. By contrast, the first and second pharyngeal arch arteries persisted beyond E12.5 in *Ednra*-null embryos. These abnormally persistent first and second pharyngeal arch arteries were likely to be the origin of the abnormal vessels from common carotid arteries, which are known to derive from the third pharyngeal arch arteries.

4. The Edn1/Ednra signaling regulates the properly-directed differentiation of neural crest cells into smooth muscle cells in pharyngeal arch arteries.

PDGFRβ is known as a marker of developing smooth muscle cells and pericytes. In E10.5 *Ednra*-heterozygous embryos, PDGFRβ-positive cells surrounded the third, fourth and sixth pharyngeal arch arteries, but not the first and second pharyngeal arch arteries. On the other hand, PDGFRβ expression was detected also around the first and second pharyngeal arch arteries in *Ednra*-null embryos. In E11.5 *Ednra*-null embryos, αSMA, a marker of differentiated smooth muscle cells, started to be expressed in the first and second pharyngeal arch arteries, in addition to PDGFRβ. These data indicate that smooth muscle cells are aberrantly developed along the first and second pharyngeal arch arteries in *Ednra*-null embryos.

Next, *Wnt1-Cre;R26R* mice were analyzed, to examine whether these aberrant smooth muscle cells in the first and second pharyngeal arch arteries derive from neural crest cells. As early as at E9.5, the forming stage of pharyngeal arch arteries, β-galactosidase-positive neural crest cells were surrounding the first and second pharyngeal arch arteries in *Wnt1-Cre;R26R* embryos, suggesting that neural crest cells are the primary source of smooth muscle cells in pharyngeal arch arteries. At E11.5, the distribution of β-galactosidase-positive neural crest cells was not different between *Ednra*-null and heterozygous embryos, but only in *Ednra*-null embryos, β-galactosidase-positive cells of the first and second pharyngeal arch arteries were positive for PDGFRβ. In E18.5 *Ednra*-null embryos, whole-mount β-galactosidase staining confirmed that neural crest-derived cells existed around the abnormal vessels

from common carotid arteries. α-SMA and β-galactosidase were coexpressed at the cells around the abnormal vessels from common carotid arteries. These results suggest that, in *Ednra*-null mice, neural crest cells abnormally differentiate into smooth muscle cells at the first and second pharyngeal arch arteries, which result in the abnormal persistence of these arteries and the formation of the abnormal vessels from common carotid arteries. This means that the Edn1/Ednra signaling is involved in appropriate deployment of neural crest-derived smooth muscle cells in pharyngeal arch arteries, which contributes to the normal patterning of thoracic great vessels.

Conclusion

The remodeling of pharyngeal arch arteries does not depend on *Dlx5/Dlx6*-mediated ventral identification of pharyngeal arches, and the Edn1/Ednra signaling regulates the properly-directed differentiation of neural crest cells into smooth muscle cells in pharyngeal arch arteries.