学 位 論 文 の 要 旨

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学　位　論　文　名　Ror2 is Required for Midgut Elongation During Mouse Development

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論 文 内 容 の 要 旨

INTRODUCTION

Gut development is characterized by rapid elongation during organogenesis, however, the underlying mechanism of the gut elongation remains unknown. It has been suggested that during the human embryonic period, the duodenum (i.e., rostral midgut) is elongated by a "convergent extension (CE)" mechanism: a common developmental phenomenon consisting of a narrowing and elongation of tissue driven by polarized cell movement. Noncanonical Wnt signaling has been implicated in polarized cell movement, and Wnt5a, which is a representative noncanonical Wnt member, was recently found to be a critical regulator of mouse gut elongation via the CE mechanism, as evidenced by the finding of shortened and widened small intestines in the absence of Wnt5a.

The receptor tyrosine kinase Ror2 acts as a receptor for Wnt5a to mediate noncanonical Wnt signaling, and it plays essential roles in morphogenesis. *Ror2*$^{-/-}$ embryos exhibit phenotypes similar to, albeit generally milder than, those of *Wnt5a*$^{-/-}$ embryos. During mouse embryogenesis, *Ror2* is expressed in various organs and regions. Although extensive studies have documented the spatio-temporal expression patterns and the pleiotrophic functions of *Ror2*, including its relation with those of *Wnt5a*, little is known about the expression pattern of Ror2 protein and its involvement in gut development. Here, we analyzed the expression pattern of Ror2 during gut development and gut phenotype of *Ror2*$^{-/-}$ embryos to elucidate roles of Ror2 in gut morphogenesis.

MATERIALS AND METHODS

Jcl:ICR (CLEA Japan, Tokyo, Japan) mouse embryos were used for the Ror2 expression analysis. Noon of the day when a vaginal plug was observed was defined as embryonic day (E) 0.5. Heterozygous (*Ror2*$^{+/-}$) mice were maintained on a C57Bl/6J background and crossed to generate *Ror2*$^{-/-}$ embryos.

Immunohistochemistry was performed using specific primary antibodies for Ror2, α-smooth muscle actin, Ret, c-Kit, E-cadherin, laminin-1, γ-tubulin, Tuj1, and phospho-PKCζ/λ (aPKC). Whole mount *in situ* hybridization was performed for analysis of *Wnt5a* expression. For proliferation assay, 5-bromo-2'-deoxyuridine-labeling was performed. The total epithelial cells of the midgut were counted based on systematic and random sampling and the length of the midgut was measured by ImageJ. The mean cell volume was yielded by dividing the total epithelial volume of the midgut by the total epithelial cell numbers. The cell division axis was calculated from the coordinates of two spindle poles in the three-dimensional epithelium. Student's *t*-test was applied to evaluate the statistical significance of the results, which was set at the level of $P<0.05$.

RESULTS AND DISCUSSION

Expression analyses of Ror2 and *Wnt5a* during gut development

The expression of Ror2 showed a region-specific pattern both in the epithelium and mesenchyme during gut development. From E10.5 to E12.5, Ror2 expression in the epithelium was the most intense, and this strong expression was the most pronounced in the rostral to middle region of the midgut. *Wnt5a*, which encodes a ligand for Ror2, was detected in the mesenchyme of the rostral stomach, caudal midgut, and rostral hindgut except for the cecum.

Defective CE mechanism suggested by a shortened and widened middle midgut of *Ror2*$^{-/-}$ embryos

The overall length of the intestines was reduced in *Ror2*$^{-/-}$ embryos as compared with that of controls at each stage from E10.5 to E18.5. In addition to the short gut, fundus of the stomach and the cecum were shortened in *Ror2*$^{-/-}$ embryos. In the *Ror2*$^{-/-}$ embryos, at E11.5, the midgut length was shorter (62%) and the outer diameter of the epithelial tube in the middle part of the midgut was larger than in the control embryos, whereas the body weight and crown-rump length (CRL) showed no significant difference. As regards control gut development from E11.0 to E11.5, the midgut length increased 1.78-fold, while the maximum diameter of the midgut tended to be reduced, in contrast, in *Ror2*$^{-/-}$ embryos, the maximum diameter did not change, while the length increased only 1.39-fold. These findings suggested that intestinal morphogenesis was disrupted in *Ror2*$^{-/-}$ embryos in terms of the proportion of elongation to diameter. The total epithelial cell counts of the midgut at E11.5 were not significantly different between *Ror2*$^{-/-}$ and control embryos. However, it was shown from cell number plotting along the rostral-caudal (RC) axis of the midgut that, in the absence of Ror2, the epithelial cells accumulated in the middle part of the midgut, corresponding to the larger diameter of the same region than in control embryos. The overall proliferation, apoptosis, and the mean cell volume of the midgut epithelium did not significantly differ between the control and *Ror2*$^{-/-}$ embryos. The distribution and the average of the cell division angles did not significantly differ between control and *Ror2*$^{-/-}$ embryos. These findings suggest that Ror2 is not involved in narrowing and elongation of the midgut via regulation of either cell proliferation, cell death, cell volume, or orientation of epithelial cell division.

In human gut organogenesis, the total midgut elongates approximately 3- to 4-fold within a short period of time (from Carnegie stage 14 to 17; approximate gestational age: 5 to 6 weeks after fertilization), whereas the CRL increases only 2-fold during the same period, which indicates that this period is critical

for midgut elongation. We previously showed that during this period, in the duodenum, diameter decreases while it elongates and there is neither a predominance of apoptosis nor an increase in cell numbers, suggesting that the epithelial cells converged toward the RC axis, resulting in gut elongation. Similarly, we found here that, during mouse gut morphogenesis from E11.0 to E11.5 (corresponding to the Carnegie stage 14 to 16 in humans), the epithelial tube diameter of the midgut had a tendency to narrow while it elongated, suggesting that the midgut elongates at least in part via the CE. These corresponding findings in humans and mice appear to verify that elongation of the midgut occurs during this critical period via the CE mechanism. The morphogenetic abnormality of the midgut found in *Ror2*$^{-/-}$ embryos suggests that the epithelial cells were unable to rearrange themselves for elongation of the tube along the RC axis. At E11.5, the reciprocal patterns of Ror2 and Wnt5a expression may create the morphogenic Wnt5a gradient for Ror2-expressing cells. It is therefore possible that with stronger Wnt5a signaling comes a greater extent of convergence of the tube in association with the gradient of Wnt5a along the RC axis. This hypothesis could explain the shape difference of the midgut between *Ror2*$^{-/-}$ and control embryos: dilatation and abrupt narrowing of the midgut in *Ror2*$^{-/-}$ embryos versus gradual narrowing along the RC axis in control embryos.

Disrupted cell polarity in an aberrant cell mass in the midgut of *Ror2*$^{-/-}$ embryos

One characteristic finding, suggesting that Ror2 may also be involved in the regulation of cell polarity was the aberrant epithelial cell clumps in *Ror2*$^{-/-}$ embryos at E11.5, which were similar to those reported in *Wnt5a*$^{-/-}$ embryos. Interestingly, a single clump per embryo was observed in a region limited to that immediately rostral to the bifurcation of the patent vitelline duct. In these cell clumps, the cells appeared to have lost their apico-basal (AB) polarity, as confirmed by the finding that phosphorylated aPKC, a marker for AB polarity, was partially absent in the apical region of these clumps.

Ror2 as a receptor for Wnt5a to mediate downstream signaling during gut development

Although the abnormal phenotypes found in the gut of *Ror2*$^{-/-}$ embryos were not as severe as those of *Wnt5a*$^{-/-}$ embryos, many similarities between the two mutants were observed in the gut phenotype, including a shortened and widened midgut, which suggest that Ror2 indeed acts as a receptor for Wnt5a to mediate downstream signaling during gut development. However, we also observed several distinct differences between *Ror2*$^{-/-}$ and *Wnt5a*$^{-/-}$ embryos, including difference of affected regions of the gastrointestinal tract, suggesting that other downstream pathways for Wnt5a signaling and/or other ligands for Ror2 exist in gut development.

CONCLUSION

We demonstrated that Ror2 was strongly and differentially expressed in the rostral and middle midgut endoderm from E10.5 through E12.5. At E11.5, *Ror2*$^{-/-}$ embryos exhibited a shorter middle midgut with a larger diameter and more accumulation of epithelial cells in the middle midgut than control embryos, while the total cell numbers remained unaltered. These findings suggest that Ror2 plays critical roles in midgut elongation via an epithelial CE mechanism. We also found AB polarity-disrupted cell clumps in the midgut epithelium of *Ror2*$^{-/-}$ embryos, suggesting that Ror2 may also be involved in the regulation of epithelial AB polarity.