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学位論文題目	Essential role of *Cables2* for gastrulation of the mouse embryo (マウス原腸胚形成における *Cables2* の役割)

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論文の要旨
Abstract of thesis

<Purpose>

The CDK5 and ABL1 enzyme substrate1 (Cables1) have multiple functions that include, axon guidance, quality control of oocyte, and tumor suppression in mice. Also several studies in human clearly showed the function of Cables1 in tumor suppression in colon and uterus. Interestingly, agenesis of corpus callosum was found in mutant mice with *Cables1* truncation but not its null mutation. Those results implied the existence of a redundant gene that might affect *Cables1* dysfunction. Cables has two family members, Cables1 and Cables2. Both mouse Cables1 and Cables2 have protein binding PXXP motif and cyclin box like domain that directly bind to several proteins (e.g. cdk3, cdk5, and c-abl). Furthermore, Cables1 and Cables2 are broadly expressed in adult mouse tissues. Although Cables2 seems to have multiple and important functions, there is very little *in vitro* study and no *in vivo* study of Cables2. Therefore, the applicant aimed to investigate Cables2 function *in vivo*. The applicant found that *Cables2* is essential for embryogenesis, especially at the gastrulation stage, and further analyzed its functional link with signaling pathway that regulates gastrulation.

<Result>

Early embryonic lethality in Cables2 KO mice

To analyze Cables2 function *in vivo*, the applicant established *Cables2* KO mouse strain from the embryonic stem cell line VG16085 produced by the VelociGene's Knockout mouse project (KOMP). VG16085 cells line carries heterozygous KO allele in which all the *Cables2* coding region is deleted. The chimeric male mice were produced by conventional aggregation method, and heterozygous *Cables2* KO (*Cables2*$^{-/+}$) mice were subsequently obtained from those progeny. Both *Cables2*$^{-/+}$ male and female mice were healthy and fertile. However, no *Cables2*$^{-/-}$ mouse was obtained by *Cables2*$^{-/+}$ intercross. This result indicates that *Cables2*$^{-/-}$ mouse is embryonic lethal, while *Cables1*$^{-/-}$ mouse showed neither embryonic lethality nor premature death. The applicant then surveyed the timing of embryonic death in *Cables2*$^{-/-}$. The embryos from *Cables2*$^{-/+}$ intercross were analyzed in five time points (embryonic day (E) 6.5, 7.5, 8.5, 9.5, 12.5). No *Cables2*$^{-/-}$ embryo was found at E12.5. From E7.5 to E9.5, *Cables2*$^{-/-}$ embryos showed abnormally small size. At E6.5, *Cables2*$^{-/-}$ embryos were present at expected Mendelian ratio with normal morphology.

The morphologies of *Cables2*$^{-/-}$ embryos were further analyzed histologically. At E6.5, there was no distinguishable difference between *Cables2*$^{-/-}$ and *Cables2*$^{+/+}$. At E7.5, three embryonic cavities, node and head fold were not seen in *Cables2*$^{-/-}$. Moreover, both the extra- and embryonic regions were smaller in *Cables2*$^{-/-}$, and the initial primitive-streak was not confirmed.

These results suggested that *Cables2* is essential for development at gastrulation stage.

Expression of *Cable2* during early mouse development

To investigate *Cables2* mRNA expression during embryogenesis, RT-PCR analyses were conducted using embryonic stem cells, blastocyst (embryonic day 3.5), and E7.5 embryos. *Cables2* mRNA was detected in every stage. Then, *Cables2* expression patterns were checked by *in situ* hybridization. The *in situ* hybridization revealed ubiquitous expression of *Cables2* mRNA in all stages of wild-type embryos analyzed (E5.5, 6.5, 7.5, 8.5, and 9.5), but not in *Cables2*$^{-/-}$.

Cell proliferation and death in Cables2 mutant embryos

Cell proliferation defect and/or excessive apoptosis may be the cause of early embryonic lethality. Therefore, the applicant investigated cell proliferation and apoptosis, by EdU and TUNEL assays, respectively. In EdU assay, the number of proliferating EdU positive cells was not significantly different between *Cables2*$^{+/+}$ and *Cables2*$^{-/-}$ at E6.5. Furthermore, the number of apoptotic cells, detected by TUNEL assay, was also the same between *Cables2*$^{-/-}$ and *Cables2*$^{+/+}$. These results indicated that lethality of *Cables2*$^{-/-}$ embryo is not due to

defective cell proliferation or enhanced apoptosis.

Impaired primitive streak formation anterior-posterior axis specification in *Cables2*$^{-/-}$

Cell differentiation and body axis formation are critical for gastrulation. The applicant performed whole *in situ* hybridization with several markers of cell differentiation and body axis formation. Because abnormal morphology of *Cables2*$^{-/-}$ embryo was observed at E7.5, the applicant analyzed those cell differentiation markers at E6.5. In this period, *Wnt3* and *T* are expressed in proximal epiblast and have critical function for primitive streak and mesoderm formation. Similar to *Cables2*$^{+/+}$, *Wnt3* and *T* was expressed in posterior side in *Cables2*$^{-/-}$ embryo. However, their expression levels were dramatically decreased. In contrast, the expression patterns of *Bmp4*, which is the marker for proximal distal axis, were not significantly different between *Cables2*$^{+/+}$ and *Cables2*$^{-/-}$. The anterior visceral endoderm (AVE) marker, *Cel1* signal was very weak in *Cables2*$^{-/-}$. *Lim1* expression in the lateral mesoderm and anterior endoderm, was also decreased in *Cables2*$^{-/-}$, and limited in distal side. These results suggest *Cables2* depletion results in impaired primitive streak and AVE formations and/or locations.

Cables2 was involved in both Wnt/b-catenin and Smad2 signaling

Since *T* expression is controlled by Wnt/β-catenin signaling, and *T* expression was abnormal in *Cables2*$^{-/-}$ embryos, the impairment of this signaling pathway was suspected. Indeed, it has been also known that Cables1 can directly bind to β-catenin and regulates the neural outgrowth via Wnt/β-catenin signaling. Therefore, the applicant analyzed the involvement of Cables2 in Wnt/β-catenin signaling by the TOP FLASH reporter assay using HEK293 cell. As expected, TOP/FOP activity was increased by *Cables2* overexpression. Moreover, the physical interaction of Cables2 and β-catenin was confirmed by co-immuno-precipitation assay. These results strongly indicated that Cables2 is involved in Wnt/β-catenin signaling.

As the *Cel1* and *Lim1* expressions are not controlled by Wnt/β-catenin signaling, *Cables2* may also have relation with other signaling cascade. Then, the applicant has focused on Smad2 signaling which plays a critical role in AVE formation and anterior-posterior axis formation. In the ARE-Luc reporter assay using HEK293 cells, the reporter signal was significantly increased by Cables2 overexpression. And the interaction of Cables2 and Smad2 was observed by co-immunoprecipitation assay, suggesting that Cables2 can also regulate the Smad2 signaling. Finally, the applicant evaluated the *Nanog* expression. This gene expression in epiblast at E6.5 embryos is dependent largely on Smad2 activity. As expected, low *Nanog* expression in epiblast was observed in E6.5 *Cables2*$^{-/-}$ embryo. These results support that Cables2 is also involved in Smad2 signaling in gastrulation.

<Discussion>

In this study, it was discovered that *Cables2* plays an essential role in embryonic development, especially in differentiation and axis formation during gastrulation. Furthermore, the applicant demonstrated that Cables2 is involved in both Wnt/β-catenin and Smad2 signaling. Further comprehensive analyses are required to clarify the molecular function of Cables2 in the above signaling cascade and explain the embryonic lethality of *Cables2*$^{-/-}$. The analyses of conditional *Cables2* knockout mice are also underway.

審査の要旨
Abstract of assessment result

【批評 Review】

This study provided the first evidence that Cabels2 is essential for the embryogenesis, especially during gastrulation. The characterization of *Cables2*$^{-/-}$ phenotypes further led to the identification of the functional links of Cables2 with the two major signaling cascades. Although the precise molecular function of Cables2 remains to be elucidated, this study proved the importance of Cables2 function and urged further comprehensive analyses of Cables2 biochemical function (ie; analyses of protein-protein interaction network). This paper also illustrated the importance to analyze Cables2 function on carcinogenesis, neurogenesis, and so on, by the use of conditional knockout mice, which is under way. Those analyses in the near future are quite promising considering the essential roles of Cables2. In this regard, this paper can be regarded as a milestone that should open a new research area in the respective research fields.

【最終試験の結果 Result】

The final examination committee conducted a meeting as a final examination on 9th May, 2017. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

【結論 Conclusion】

Therefore, the final examination committee approved that the applicant is qualified to be awarded a Doctor of Philosophy in Human Biology.