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学位論文題目	A Novel Isoform of Vinexin, Vinexin γ, Regulates Sox9 Gene Expression through Activation of MAPK Cascade in Mouse Fetal Gonad
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論文内容の要旨

In mammals, sexes are determined by the presence or absence of *Sry*, the sex determining gene on the Y chromosome. Early gonads are morphologically identical between XX and XY individuals until embryonic day 11.5 (E11.5). However, through its transient expression from E10.5 and E12.5, *Sry* initiates the developmental processes necessary to shape the XY indifferent gonad into a testis. Thereafter, multiple transcriptional regulators such as *Ad4BP/SF-1*, *Sox9*, *M33*, *Wt-1*, *Gata-4*, and *Dax-1* function to develop sexually differentiated gonads. However, it is little known about these molecular mechanisms.

To investigate the role of Ad4BP/SF-1 during mouse gonadal development, we isolated proteins that interact with Ad4BP/SF-1 by yeast two-hybrid screening with a library prepared from E11.5-13.5 mouse fetal gonads. Vinexin was one of the clones isolated by the screening. Two isoforms of Vinexin, Vinexinα and β had previously been identified, however, the clone isolated in this study was distinct from those two isoforms in the 5' region. Thus, this novel isoform was tentatively designated Vinexin γ.

To investigate the expression profile of Vinexin γ, whole-mount *in situ* hybridization was performed with mouse fetal gonads. *Vinexin γ* expression was evident from E11.5 in both sexes. Moreover, *Vinexin γ* was detected in only the cDNA prepared form the somatic cells, but not germ cells of both sexes. The expression of Vinexin was examined further with a polyclonal antibody raised to a recombinant Vinexin γ. In the E12.5 fetal gonads, Vinexin γ and β were detected, whereas Vinexin α was not detected. The expression of Vinexin in the developing gonads was examined immunohistochemically. Vinexin expression was observed in Sertoli cells of the XY gonads and in the somatic cells of the XX gonads. In addition, Vinexins were localized in the cytoplasm but not in the nuclei at all stages examined.

Recently, it was reported that Vinexin β regulates EGF-induced phosphorylation of c-jun N-terminal kinase (JNK) and anchorage-dependent phosphorylation of ERK2. Therefore, we examined whether Vinexin γregulates the MAPK cascade in a mouse embryonic fibroblast cell line, C3H10T1/2. Phosphorylation of ERK and MEK was found to be up-regulated significantly after the transfection of Vinexin γ while that of c-Raf was not up-regulated. Since Vinexin γ was also implicated in regulation by the MAPK cascade, we examined the interaction between Vinexin γ and components of the MAPK cascade. Vinexin γ interacted with endogenous ERK and c-Raf but not MEK. The interaction with c-Raf appeared to be stronger than that with ERK.

It was previously reported that Fgfs was implicated the MAPK cascade in regulation of *Sox9* gene expression in chondrocytes. Thus, we hypothesized that Vinexin γ is involved in regulation of *Sox9* gene expression through interaction with components of the MAPK cascade. Based on this hypothesis, Vinexin γ increased *Sox9* mRNA levels. Conversely, when the cells were treated with a specific inhibitor of MEK activation, U0126, the reagent markedly inhibited up-regulation of *Sox9* gene expression by Vinexin γ. Phosphorylation of ERK was also inhibited. These observations strongly suggested that Vinexin γ regulates *Sox9* gene expression by activating the MEK-ERK cascade.

To investigate the functions of Vinexin γ *in vivo*, we generated *Vinexin γ* gene-disrupted mice. We generated a Vinexin γ-specific disrupted mice by deleting the *γ* form-specific first exon together with the upstream region. This Vinexin γ-specific

disrupted mice were produced successfully, however these mice were obtained at the predicted Mendelian ratios and were fertile in both sexes. Moreover, the XX and XY fetal gonads of the disrupted mice were morphologically normal.

In order to examine whether MAPK activation occurs in mouse fetal gonads, Western blot analyses with mouse fetal gonads were performed using antibodies to phosphorylated forms of MAPK (ERK, JNK, and p38). Interestingly, ERK was phosphorylated, whereas JNK and p38 were not phosphorylated in the fetal gonads at E12.5. We then examined ERK phosphorylation in the Vinexin $\gamma^{-/-}$ fetal gonads. ERK phosphorylation was decreased in the Vinexin $\gamma^{-/-}$ XY gonads when compared to the Vinexin $\gamma^{+/-}$ XY gonads at E12.5. In contrast, there was no significant difference in ERK phosphorylation between the Vinexin $\gamma^{+/-}$ and Vinexin $\gamma^{-/-}$ XX gonads. These sexually distinct effects on ERK phosphorylation strongly suggested that the phosphorylation mediated by Vinexin γ occurs in the male but not in the female gonad, even though Vinexin γ is expressed in the gonads of both sexes.

Since ERK phosphorylation was shown to lead to up-regulation of *Sox9* expression, *Sox9* expression was expected to be affected by *Vinexin γ* gene disruption. Thus, *Sox9* expression in the gonads was examined by whole-mount *in situ* hybridization. *Sox9* expression was affected in the Vinexin $\gamma^{-/-}$ XY gonads at E12.5. However, the decreased level of *Sox9* varied among the Vinexin $\gamma^{-/-}$ XY gonads. Indeed *Sox9* expression was either similar to the wild-type, decreased modestly, or decreased severely. However, *Sox9* expression did not completely disappear from any of the Vinexin $\gamma^{-/-}$ XY gonads. Interestingly, the decreased *Sox9* expression was observed with higher frequency at E11.5, and with lower frequency at E13.5 and E14.5. *Sox9* expression eventually recovered during the late fetal period. As a control for altered *Sox9* expression in the gonad, *Sox9* expression was investigated in the limb buds where Vinexin γ is not expressed. As expected, no difference in *Sox9* gene expression was observed between the Vinexin $\gamma^{+/-}$ and Vinexin $\gamma^{-/-}$ hind limb buds.

In conclusion, we identified a novel isoform of Vinexin, Vinexin γ and characterized it as a scaffold protein for the MAPK cascade. Through regulating the activation of MEK-ERK via interaction with c-Raf, Vinexin γ is implicated in *Sox9* gene expression. The gene disruption study revealed that Vinexin γ regulates the process of testis differentiation through modulating the MAPK cascade.

論文審査結果の要旨

遺伝子破壊マウスの解析結果から、核内受容体 Ad4BP/SF-1 は生殖腺や副腎皮質の形成に必須の因子であることが明らかになっている。本研究では Ad4BP/SF-1 と相互作用する因子を two-hybrid スクリーニングによって単離し、その機能の解析を通じ、生殖腺の形成メカニズムを明らかにすることを目的とした。本研究においては、既に単離されていた相互作用因子の中で、胎仔生殖腺に発現し、３個の SH3 ドメインを有する Vinexin に着目した。この因子にはαとβ isoform の存在が報告されていたが、今回新たにγ form が同定された。生殖腺の形成過程における Vinexin γの機能を調べるため遺伝子破壊マウスを作製したところ、このマウスの生殖腺は雌雄ともに正常で妊性を有していた。また、その他の組織においても顕著な異常は認められなかった。

哺乳類の生殖腺は胎仔期に性的に未分化な生殖腺から雌雄生殖腺へと性分化する。この過程で Y 染色体上に存在する SRY 遺伝子が精巣決定因子として機能することが知られている。後にこの遺伝子の発現は SOX9 遺伝子の発現誘導を通じ、精巣セルトリ細胞の細胞系譜の決定を行っている。Vinexin γ遺伝子破壊マウスにおいて各種生殖腺のマーカー遺伝子の発現を検討したところ、Sox9 遺伝子の発現が減少していた。この結果は Vinexin γが Sox９遺伝子の転写制御に関与することを示唆したことから、そのメカニズムの解析を行った。その結果 Vinexin γは c-Raf と ERK との相互作用を通じ、MEF と ERK のリン酸化を促進することで ERK カスケードの調節を行っていることが明らかになった。実際に Vinexin γ遺伝子破壊マウスの雄胎仔生殖腺では ERK の活性化が抑制されていた。また、培養細胞を用いた実験から ERK カスケードの活性化が Sox9 遺伝子の転写を上昇させた。以上の結果から、Vinexin γは生殖腺の性決定の後に、ERK カスケードの調節を通じて雄生殖腺特異的に発現する Sox9 遺伝子の転写を調節することで、生殖腺の雄化プロセスに関与することが明らかになった。

本研究は生殖腺の性分化過程における ERK カスケード関与をはじめて示したのもで、審査委員会は学位論文として十分な内容を有すると判定した。

本研究内容について口頭発表を行わせた後、審査委員が研究内容及び実験手法、関連する周辺知識について試問を行った。その結果、申請者が研究内容の把握、考察、基礎知識等、学位に値する能力を備えていると判断した。また、申請者は国際誌に論文を発表しており、英語に関する能力も十分備えている。以上より申請者が学位授与に相応しい水準に達しているものと判断した。