氏　　名	佐藤優子
学位（専攻分野）	博士（理学）
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学位論文題目	Importance of forkhead transcription factor Fkhl18 for development of testicular vasculature
論文審査委員	主　査　教授　井口　泰泉 教授　髙田　慎治 教授　小林　悟 教授　佐々木　裕之 教授　諸橋　憲一郎（九州大学） 教授　中西　義信（金沢大学）

論文内容の要旨

Mammalian sex is determined by differentiation of the gonad (testis or ovary), which governs phenotypic sex through the production of hormones. Initially, embryonic gonads develop as bipotential gonad, and its sexual differentiation is genetically controlled: depending on the presence or absence of the Y chromosome. In the developing testis, male-specific patterning of the vasculature is induced by *Sry*. Following the expression of *Sry*, endothelial cells are recruited vigorously to the testis from mesonephros and a large artery is formed at the coelomic surface at around embryonic day (E) 12.5 (coelomic vessel). Thereafter, the vessel branches from the coelomic vessel and extends progressively between testicular cords. In contrast, no such active cell migration is observed in the developing fetal ovary. This male-specific vascular system that develops during fetal life is required for efficient export of testosterone from the testis to masculinize the embryo.

Forkhead (Fox) transcription factors carry a winged helix DNA-binding domain that share homology with their founding member forkhead protein in *Drosophila*. Phylogenic analysis of the forkhead domain consisting of highly-conserved 100 amino acids led to placement of the family members into 20 subclasses, *FoxA* to *FoxS*. Fox proteins bind to consensus sequences, RYMAAYA (R=A or G; Y=C or T; M=A or C), as a monomer. Regions other than the conserved domain vary in terms of sequence and function. Some members act as transcriptional activators while others as repressors. Probably as transcriptional regulators, *Fox* genes are thought to play a variety of roles in fetal and adult tissues and mutations in *FOX* genes have been linked to human diseases. *Fkhl18*, a member of the *Fox* family, was originally identified by low-stringency screening of mouse and human genomic libraries. *Fkhl18* has low homology to other members of the Fox family, and is categorized under the *FoxS* subclass. However, its expression and function remain to be examined. In the present study, she demonstrated that *Fkhl18* was expressed in periendothelial cells and Sertoli cells of the developing fetal testis. She then generated the *Fkhl18* knockout (KO) mouse to examine the physiological function of the gene product. Interestingly, the KO fetuses displayed affected testicular vasculature, suggesting that *Fkhl18* was involved in development of the fetal testis vasculature system.

Fkhl18 KO mice displayed the following testicular abnormalities during fetal life; 1) accumulation of blood cells in the central part of the fetal testis, 2) presence of gaps, measuring 100-400 nm in diameter, between endothelial cells, allowing leakage of injected carbon ink from the testicular vessels, and 3) aberrant apoptosis of periendothelial cells. These features strongly suggest the importance of *Fkhl18* expression in the periendothelial cells for development of the testicular vascular system through direct and indirect regulation of the functions of periendothelial and endothelial cells, respectively. The indirect function of *Fkhl18* indicates a functional interaction between endothelial and periendothelial cells. The importance of interactions between the two cell types for vascular maturation has been examined by gene knockout studies of signal transductions: *angiopoietin-1/TIE-2 receptor and platelet-derived growth factor (PDGF)-BB/ PDGF receptor β (PDGFRβ)*. Unlike these KO mice, recruitment of periendothelial cells did not seem to be affected in the fetal testes of *Fkhl18* KO mice. Interestingly, however, marked apoptosis of periendothelial cells was observed; with resultant focal and transient loss of periendothelial cells. Since the expression of *Fkhl18* was

not detected in endothelial cells, the structural defect induced in endothelial cells possibly resulted from weakened interaction with the affected or decreased periendothelial cells.

To further investigate the molecular mechanisms underlying proapoptotic effect of *Fkhl18*, she demonstrated electrophoretic mobility shift assay (EMSA) and reporter gene assay. Reporter gene assays revealed that Fkhl18 suppressed transcription mediated by FoxO3a and FoxO4. Since EMSA showed that Fkhl18 had ability to bind to consensus DNA binding sequence for *Fox*, it potentially repressed transcription by competing for binding sites with other Fox proteins. Considering the suppressive function of Fkhl18, it is interesting to note that *FoxOs* mediate proapoptotic gene expression. For example, overexpression of *FoxOs* resulted in apoptosis through direct induction of proapoptotic genes: *tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)* in prostate cancer, *bim (Bcl-2 interacting mediator of cell death)*, and *FasL*. Based on the results published so far, she hypothesized that the marked apoptosis of periendothelial cells in *Fkhl18* KO testes was caused by defective proapoptotic gene transcription, which was normally attenuated by *Fkhl18*. As expected, *Fkhl18* suppressed transcription from *FasL* gene promoter in cultured smooth muscle cells prepared form bovine blood vessels.

In the present study, she focused on the function of *Fkhl18* during blood vessel formation of the fetal testis; blood vessel development in the ovary remains to be investigated. Likewise, she has not examined whether the blood vessels in tissues other than the gonads are affected by *Fkhl18*. Considering that *Fkhl18* is expressed in periendothelial cells of other tissues, the defects seen in the fetal testis could be also seen in other tissues. However, obvious accumulation of blood cells was not observed in any tissues other than the testis, strongly arguing against a major defect of blood vessel development in these tissues. Together with the highest expression of *Fkhl18* in the developing testis, it is conceivable that *Fkhl18* plays a unique role in the development of the testicular vasculature system.

論文の審査結果の要旨

脊椎動物の性は生殖腺の性によって決定される。遺伝的に性が決定される哺乳類の場合、XY の性染色体を持つ個体に精巣が、XX の性染色体を持つ個体に卵巣が分化する。次いで、精巣と卵巣で合成される性ステロイドホルモンの作用により、個体全体の性分化が進むのである。従って、脊椎動物の性分化を理解するにあたって、生殖腺の性分化メカニズムの理解は欠かせない。これまでに、性的に未分化な生殖腺から精巣が分化するにあたり、精巣に特徴的な血管形成が進むことが知られていた。本研究は、血管形成に必要とされる転写因子の精巣形成過程における機能を調べたものである。

申請者は、Fork-head 型転写因子 Fklh18 が胎仔生殖腺の血管に発現しており、この発現が性差を示すことを見いだした。形成途上の血管は血管内皮細胞とそれを取り巻く periendothelial 細胞によって構成されているが、このいずれの細胞に発現しているかは in situ hybridization では決定できなかったので、本遺伝子を用いてトランスジェニックマウスを作製することで periendothelial 細胞に発現することを明らかにした。本遺伝子の機能の解明を目的に遺伝子破壊マウスを作成したところ、生殖腺の構造や生殖能力に顕著な異常は見いだせなかったものの、胎仔精巣の血管構造に異常が認められた。この遺伝子破壊マウスの精巣血管の内皮細胞には野生型に比べて細胞死の出現頻度があがっており、血管の気密性が障害されていた。

Fork-head 型転写因子が細胞死を調節する Fas ligand 遺伝子の発現に関与することが知られていたので、Fklh18 が同様の機能を有するかについて調べた。その結果、本因子は他の活性化型 Fork-head 型転写因子の転写を抑制することで、通常の精巣血管形成時に細胞死を抑えていることが示唆された。

以上の研究成果は精巣の血管形成過程における Fork-head 型転写因子の機能を初めて調べたものであり、学位の取得に値すると審査員全員が判断した。