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論文内容の要旨

Germ cells have a capacity to undergo meiosis, a unique form of cell cycle halving the genetic material, and ultimately differentiate into sperms or oocytes. Thus, these haploid cells are crucial for transfer of genetic information from one generation to the next without affecting chromosome number. A fundamental question in reproductive biology relates to how these cells determinate their fate and commit to either spermatogenesis or oogenesis. Interestingly, the decision of their sexual fate is independent of their XX or XY chromosomal constitution. Instead, sexual fate of germ cells is determined by the cues from their environment. In the mouse, exposed to high level of retinoic acid (RA), germ cells in fetal ovaries embark on meiosis, and simultaneously, these germ cells commit to oogenesis, expressing several essential factors for follicular development. In contrast, germ cells in testes do not enter meiosis until after birth. They are protected from meiotic entry by *Cyp26b1*, encoding an enzyme that directly degrades RA. Meanwhile, an essential intrinsic factor Nanos2 is expressed in XY germ cells and promotes male differentiation. It is considered that the precise regulation of RA level in fetal gonads is important for specification of germ cell fate. However, it is still unclear what signals directly induce *Nanos2* expression in fetal testes. Moreover, simultaneous progression of meiosis and oogenesis in XX germ cells had been uncoupled, which prevented us to unveil the mechanism whereby oogenesis is regulated. In my studies, via functional analysis of transforming growth factor beta (TGFβ) signaling pathways: nodal/activin and bone morphogenetic protein (BMP) signalings in fetal gonads, I proved that nodal/activin signaling is responsible for both *Nanos2* induction and meiotic suppression of XY germ cells in testes, while BMP signaling determines sexual fate of XX germ cells in ovaries independently of RA. My thesis is composed of three parts.

In the first part (Chapter I), I described the identification of nodal/activin as a

key regulator of the male germ cell fate. A male-specific expression pattern of genes involved in nodal/activin signaling implied an important role of this signaling pathway in testicular differentiation. Indeed, inhibition of nodal/activin signaling *ex vivo* using specific inhibitors drives male germ cells into meiosis and causes failure of *Nanos2* induction. Moreover, I proved that nodal/activin played dual roles in both suppression of meiosis and the induction of *Nanos2*, because the suppression of meiosis by an RA receptor antagonist could not rescue the elimination of *Nanos2* expression caused by the loss of nodal/activin signaling. Nodal and activin-A bind their receptors that subsequently activate the Smad2/3/4 transcriptional machinery and trigger expression of target genes. Because these receptors are ubiquitously expressed, it is possible that nodal/activin-A directly works on germ cells or indirectly acts via activating somatic cells, which may send a secondary signal to promote male germ cell differentiation. Induction of *Nanos2* expression was observed when activin-A was added to purified male germ cells, implying the direct regulation of nodal/activin-A to germ cells. In addition, when Smad4, a mediator of nodal/activin signaling, was specifically deleted from male germ cells, some male germ cells entered meiosis and failed to express *Nanos2*. These phenomena were never observed in control testes, suggesting that the activation of nodal/activin signaling through Smad proteins is required for spermatogenesis. However, only a small part of germ cells entered meiosis, implying a Smad4-independent pathway also contributes to regulate germ cell fate. Moreover, I clarified that the initiation of nodal/activin signaling requires Fgf9 signaling, which is secreted from pre-Sertoli cells under the control of SRY, the sex determinant of mouse testes. Therefore, nodal signaling is specifically initiated in XY germ cells.

In the second part (Chapter II), I investigated how nodal/activin signaling suppresses meiosis and induces *Nanos2* expression, by focusing two factors p38 mitogen-activated protein kinase (MAPK) and OTX2 transcription factor, because (i) these two factors are specifically expressed in male germ cells; (ii) during the

formation of anterior and posterior body axis, these two factors are regulated by nodal signaling. Indeed, OTX2 expression level decreased after nodal inhibitor treatment, indicating the regulation of OTX2 expression by nodal signaling in germ cells. To examine the role of OTX2 in male sexual differentiation, I deleted *Otx2* from fetal testes. After the deletion of *Otx2*, activation of *Nanos2* expression was temporally suppressed and recovered in later stage, suggesting the loss of OTX2 was rescued by some other factors. Indeed, I detected the higher expression of *Otx3*, which have the same binding site with OTX2, in mutant testes. Then, using a specific inhibitor I found that the suppression of p38 MAPK *ex vivo* in fetal testes caused ectopic meiotic entry of male germ cells. In addition, I found that the expression level of *Nanos2* decreased after the inhibitor treatment and the downregulation of *Nanos2* was vanished when these testes were exposed to RA receptor antagonist. These results suggested that p38 MAPK was merely permissive but not instructive for the *Nanos2* expression, in which p38 protected male germ cells from entering meiosis by the RA signaling. Unexpectedly, the expression of pp38 persisted even nodal signaling was suppressed, implying that there is an independent pathway to activate p38 signaling. I conclude that nodal/activin signaling regulates male germ cell fate through OTX2-dependent induction of *Nanos2* expression. I also like to propose that both of these two pathways are essential for the suppression of RA signaling, although the activation of p38 is independent of nodal.

In the last part (Chapter III), I report that BMP signaling plays dual roles in sex determination of XX germ cells. BMP signaling regulates meiotic progression after RA-dependent meiotic initiation and promotes XX germ cell fate independently of RA. To clarify the functions of BMP signaling, Smad4, a co-activator of both nodal/activin and BMP signaling pathways was conditionally deleted before sex determination of germ cells. Germ cells in the mutant ovaries fail to form double strand breaks (DSBs) during meiosis. The expression levels of several genes involved in DSBs were

significantly decreased after the disruption of BMP signaling. Interestingly, the disruption of DSBs was not ascribed to the loss of RA signaling, because the expression of *Stra8*, one of the direct targets of RA, is normally initiated. Notably, exogenous addition of RA into *Smad4* mutant ovaries could not rescue the deficiency. Considering that nodal/activin signaling is not activated at this stage in ovaries, the phenotype observed in mutant ovaries was imputed to the loss of BMP signaling.

Furthermore, the disruption of BMP signaling in the fetal ovaries causes XX germ cells to maintain pluripotency genes, slight upregulation of male-specific genes (*Nodal* and *Nanos2*) and fail to activate *Figla* and *Nobox*, which are essential factors for follicular development. Therefore, the loss of BMP signaling resulted in incomplete sex reversal of XX germ cells. Interestingly, the upregulation of nodal signaling was not accompanied with the enhancement of Fgf9 signals, suggesting an indirect role of Fgf9 in the initiation of nodal signaling by the suppression of BMP signaling. Importantly, the initiation of sex reversal of XX germ cells was not caused by meiotic failure, because the suppression of meiotic initiation by the treatment of the RA receptor inhibitor did not lead to sex reversal. I conclude that BMP regulates oogenesis by impeding male pathway, and instead activating genes required for oogenesis. The initiation of sex reversal occurs independently of meiotic entry in the *Smad4* mutant ovaries, indicating that BMP but not RA regulates XX germ cell fate. Finally, by the treatment of *Smad4* mutant ovaries with the RA receptor inhibitor, I proved that incomplete sex reversal of XX germ cells was ascribed to RA judging by the successful induction of complete sex reversal of XX germ cells in *Smad4* mutant ovaries after the suppression of RA signaling.

Taken together, my study revealed that TGFβ signaling pathways are essential for sex determination of mammalian germ cells. In ovaries, accompanying with meiotic initiation by RA, BMP instructs oogenesis by inducing genes required for the formation of primordial follicle and meiotic progression, and also suppressing the

male-specific nodal signaling. BMP might directly act on germ cells or indirectly induce a secondary signal from somatic cells to regulate germ cell fate. In contrast, XY germ cells are protected from RA by an enzyme CYP26B1 that is regulated by Fgf9/Sox9 signaling. Meanwhile, germ cell intrinsic nodal signaling promotes spermatogenesis by inducing *Nanos2* expression. The disruption of RA and BMP signalings in fetal ovaries is sufficient for the upregulation of nodal signaling and for the induction of complete sex reversal of XX germ cells indicating spermatogenesis is a default pathway for germ cells. However, the mechanism whereby nodal signaling is initiated is still unknown. It might be a cell autonomous event in germ cells or be triggered by a signal from somatic cells which is suppressed by BMP signaling in fetal ovaries. These results provide important information to understand the mechanisms guiding sex determination of germ cells. Moreover, TGFβ members are highly conserved among animals, implying similar mechanisms might also exist in other animals. Therefore, the mechanism of sex determination in germ cells might be far more conserved than we previously considered.

博士論文の審査結果の要旨

マウス生殖細胞の雄性分化は、生殖巣のセルトリ前駆細胞で性決定遺伝子 *Sry* と *Sox9* が発現し、その下流にある Fibroblast growth factor 9 (FGF9) が分泌されて、誘導されることが知られている。また、呉君の所属研究室では RNA 結合タンパク NANOS2 がマウスの生殖細胞で発現し、減数分裂を抑制して雄性化を引き起こすことを見出している。呉君は FGF9 が NANOS2 を誘導するシグナル伝達経路に興味を持ち、以下の解析を行った。

はじめに、生殖細胞が雄性化する際に発現する遺伝子を見つけることを目的として、胎生 12.5 日 (E12.5) から E13.5 特異的に生殖細胞で発現する遺伝子のマイクロアレイ解析を行った。そこで見つかった遺伝子に対して、発達段階の異なる精巣や単離した生殖細胞における発現解析、生殖巣組織培養における阻害剤や活性化剤の添加実験、シグナル伝達因子 *Smad4* や *Nodal* の生殖細胞特異的なコンディショナルノックアウト(cKO)マウスを用いた解析を進め、FGF9 の下流で TGFβ系因子の NODAL/ACTIVIN A が働くことを見つけた。生殖巣の *Nodal* mRNA は雄の生殖細胞中でのみ発現し、*Nodal* 遺伝子を性分化時期に欠失させた cKO マウスでは *Nanos2* mRNA の発現が減少することから、生殖細胞中における *Nanos2* 発現誘導因子は NODAL と結論した。

次に、生殖細胞における NODAL の下流因子の解析を進め、TGFβ系因子の阻害剤により発現に差のある遺伝子をマイクロアレイで解析した。その結果、TGFβ系の下流の因子として知られる OTX2 を見つけた。その cKO マウスの解析から、OTX2 は *Nanos2* mRNA の発現に OTX3 と相補的に機能することが示唆された。さらに、NODAL の下流にあることが知られている p38 MAPK についても解析を進め、減数分裂を制御する *Nanos2* の発現を制御すること、*Nanos2* がない場合でも減数分裂を抑制することも見つけた。

また、雄性化における FGF9-NANOS2 の解析に加えて、*Smad4* cKO マウスを用いて XX 生殖細胞の雌性化における BMP の役割についても解析を進めた。SMAD4 は TGFβ系の共通のシグナル伝達因子であり、その不活化は XX の生殖巣においては BMP シグナルの阻害を意味する。生殖巣組織培養における阻害剤の添加実験と組み合わせて解析した結果、BMP シグナルは XX 生殖細胞における減数分裂の double strand break に必要であり、それが阻害されることで雄型の生殖細胞の特徴を示すことを見つけた。XX 生殖細胞の減数分裂の開始は retinoic acid による *Stra8* 遺伝子の活性化により引き起こされることがすでに知られているが、BMP シグナルは *Stra8* の発現に影響を及ぼさないことなどから、STRA8 の経路とは独立に働いていることが示唆されている。

以上の成果は豊富な実験データをもとに示されており、マウス生殖細胞の性分化の分野において優れた研究成果である。雄性化で FGF9 の下流に NODAL/ACTIVIN A が働くことを示した意義は大きく、また、雌性化に BMP が働くとの知見も注目を集める研究成果となるであろう。以上の理由から、呉君の博士論文は博士号授与の要件を満たすと審査員全員一致で判断した。