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学 位 論 文 題 目　The molecular dissection of NANOS2 function required for male germ cell differentiation

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博士論文の要旨

氏　　名　　島田龍輝

論文題目　　The molecular dissection of NANOS2 function required for male germ cell differentiation

Germ cells are highly specialized cells capable of transmitting genetic information to the next generation, which is accomplished by sexually differentiated gametes, sperm and eggs. Therefore, understanding the precise mechanism of sex-specific differentiation of germ cells is an important issue in reproductive biology.

In mice, sexually bi-potent germ cells begin the sex-specific differentiation after colonizing gonads, whereupon male germ cells stop the mitotic cell cycle at the G0 phase and female germ cells initiate meiosis. In male germ cell development, the evolutionarily conserved RNA-binding protein, NANOS2 is essential because NANOS2-null germ cells exhibit several abnormalities; the failure of male-type protein expression, resumption of mitosis, initiation of meiosis and escape from seminiferous tubules. Therefore, elucidating the molecular function of NANOS2 is vital to understanding male germ cell development. As NANOS2 is known to act as a post-transcriptional suppressor of its target mRNAs, identification of NANOS2-targets is an important issue. Although efforts have been made to identify NANOS2 targets, only a few relevant RNAs have been reported.

In my thesis study, I used two distinct strategies to analyze NANOS2 function. One was the conditional knock out (cKO) analysis to examine the NANOS2 function as an RNA-regulatory molecule. NANOS2 localizes to processing bodies (P-bodies), which are cytoplasmic ribonucleoprotein complexes involved in the suppression of mRNAs, suggesting that the P-body localization of NANOS2 is required for its

functionality. However, this remains unclear.

To address this issue, I tried to eliminate P-bodies from male germ cells. For this purpose, I deleted a core component of P-bodies, DDX6. As DDX6 is ubiquitously expressed in embryos, I planned to delete it only in male germ cells using a cKO strategy. In general, producing cKO mice is time consuming. To overcome this problem, I employed a new method, chimera analysis. For this analysis, I established an ES-cell line containing a germ cell-specific and tamoxifen-inducible Cre recombinase and a reporter. I then generated *floxed-Ddx6* allele homozygously into this ES-cell line. By generating chimera with floxed-*Ddx6* ES-cells and injecting tamoxifen, I obtained *Ddx6*-cKO germ cells quickly. I found that the deletion of DDX6 resulted in P-body loss without affecting NANOS2 expression. Even the presence of NANOS2, DDX6-null germ cells exhibited similar abnormalities to NANOS2-null germ cells, i.e., the resumption of mitosis and the lack of male-type gene expression. These results suggested that the localization of NANOS2 to P-bodies is required for NANOS2 to regulate its target RNAs. On the other hand, other abnormalities observed in NANOS2-null germ cells, such as initiation of meiosis and germ cells escaping from the seminiferous tubules, were not observed in DDX6-null germ cells. Therefore, NANOS2 functions can be separated into P-body-dependent and -independent functions.

Another strategy was single-cell transcriptomic analyses to explore possible target RNAs. NANOS2 has been considered to act as a male determinant because NANOS2-null male germ cells abnormally initiate meiosis, similar to female germ cells, and fail to express male-marker genes. This idea is supported by the ectopic expression of NANOS2 in female germ cells that results in the induction of male-type gene expression. However, the target responsible for the promotion of male differentiation has not been identified due to the complexity of NANOS2-targets and the limitations of applicable technology.

To address this issue, I took advantage of the cutting-edge technology of single-

cell RNA-sequencing (scRNA-seq) to analyze NANOS2 function more precisely during male germ cell development in the second part of my thesis. I found that germ cells start sexual differentiation before *Nanos2* expression. This result is supported by the analyses of *Nanos2*-KO germ cells, which retained male properties; however, it is in contrast to the idea that NANOS2 functions as a male determinant. Further analysis revealed that several male-type genes increased expression in the E14.5 *Nanso2*-KO cells compared to E13.5 KO cells. These data indicate that NANOS2 is not the male determinant and other pathways also regulate the male-type differentiation. By correlating gene expressions and the cell cycle status, I found that the earliest function of NANOS2 was the induction of cell cycle arrest. To clarify the mechanism of cell cycle regulation by NANOS2, I analyzed the expression of cell cycle regulators. As a result, NANOS2 was suggested to regulate the cell cycle by suppressing mTORC1 signaling. In addition, I found that another cell cycle suppressor, p38 MAPK signaling, was active in *Nanos2*-KO cells, raising the possibility that parallel signaling cascades are involved in cell cycle arrest in male germ cells. Lastly, by comparing scRNA-seq data with *Ddx6*-KO RNA-seq data, I identified possible targets involved in P-body-dependent and -independent NANOS2 pathways. Thus, my thesis study provides the new insights into the regulatory mechanism of the male-type differentiation of germ cells.

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博士論文審査結果

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Title
論文題目　The molecular dissection of NANOS2 function required for male germ cell differentiation

RNA 結合タンパク NANOS2 は雄の生殖細胞でのみ発現し、特定の mRNA の発現を抑制することで生殖細胞のオス化に働くと考えられている。しかしながら、NANOS2 制御下の分子カスケードはまだ不明な点が多い。島田さんは NANOS2 の生殖細胞のオス化に果たす機能を解明することを目的に、異なる２つのアプローチで研究を進め、博士論文もそれに対応した２部構成となっている。

第１章では、NANOS2 が mRNA 分解酵素複合体 CCR4-NOT と相互作用することや、細胞質顆粒の Processing (P)-body に局在することから、NANOS2 の機能発現には P-body が必要であると仮定し、P-body が NANOS2 の機能に果たす役割を調べた。P-body の主要要素の DDX6 に着目して生殖細胞でこの遺伝子の条件的ノックアウト（cKO）を行い、また、ノックアウト細胞の表現型解析を迅速に行うために、遺伝子操作した ES 細胞と正常胚のキメラ個体において直接 cKO を誘導する方法を開発して解析を進めた。その結果、目的通りにキメラ個体で *Ddx6* 欠損生殖細胞が得られ、その細胞には P-body が形成されず、NANOS2 の集積も起こっていないことが認められた。そして、これらの *Ddx6* 欠損生殖細胞では、*Nanos2* 欠損細胞と同様に、*Dazl* の発現上昇、*Dnmt3l* の発現欠失、細胞周期の再開が起こることがわかった。一方で、*Nanos2* 欠損細胞で認められる、*Stra8* の発現と精細管からの離脱は *Ddx6* 欠損細胞では認められないことがわかった。以上の成果は、NANOS2 に P-body に依存的、非依存的な機能が存在することを示唆するものである。

第２章では、NANOS2 のターゲット候補を包括的に把握することを目的に、生殖細胞が性的に未分化な胎生 11.5 日（E11.5）から性決定を受けた E15.5 までの生殖巣の single-cell RNA-seq (scRNA-seq) 解析を行なった。その結果、体細胞では E11.5 で、生殖細胞では E12.5 で性分化が認められ、体細胞の性分化が生殖細胞の性分化をリードするというこれまでの知見とよく一致した。一方、E13.5 と E14.5 の *Nanos2* KO の生殖細胞ではオス分化の特徴が見つかり、これはこれまで推測されていた NANOS2 が生殖細胞のオス化決定因子であることを覆す結果であった。さらに、*Nanos2* KO 生殖細胞において細胞周期が停止する時に p38 MAPK 活性が高まることを見つけ、NANOS2 とは独立に生殖細胞周期調節機構があることや、第１章で見られた NANOS2 下流の DDX6 非依存的な機能に関連して、ミトコンドリア活性関連遺伝子や細胞骨格フィラメント関連遺伝子があることを見つけている。マウスの性分化段階の生殖細胞の特徴を１細胞レベルの包括的遺伝子発現解析で示した価値の高い成果である。

以上の島田さんの研究成果は、マウス胎児期の生殖細胞の発達において既存の知識

を覆すなど重要な新規知見を与えるものであり、博士論文として質量ともに十分な内容を含んでいる。前半部の研究については、すでに査読つきの国際学術雑誌に公表されており、遺伝学専攻の博士授与の水準を十分に満たしていると、審査員全員一致で判断した。