様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科研費 KAKENHI

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研究課題名（和文）Identification of novel testis-specific long noncoding RNAs: a new molecular basis of infertility

研究課題名（英文）Identification of novel testis-specific long noncoding RNAs: a new molecular basis of infertility

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交付決定額（研究期間全体）：（直接経費）　　3,100,000円

研究成果の概要（和文）：Long non-coding RNAs (lncRNAs)は遺伝子発現における重要な調節因子である。しかし、精巣特異的lncRNAsの発現と機能についてはあまり知られていない。本研究ではマウスの精巣特異的なlncRNAのを発見し、これらのlncRNAを（1）特徴付け、（2）機能解析を行った。結果として、 1700108J01Rik及び 1700101022Rikが精巣特異的lncRNAとして同定された。これらは、マウスの精子形成中に異なるステージにおいて細胞特異的な発現パターンを示した。今後はこれらのlncRNA に関してin vivo でレンチウイルスを介した機能解析を行う予定である。

研究成果の学術的意義や社会的意義
This study provides useful information about testis-specific lncRNAs in mouse spermatogenesis. It would apply (1) for future investigation of testis-specific lncRNA involved in spermatogenesis and testicular functions, and (2) for better understanding of a new molecular basis of male reproduction.

研究成果の概要（英文）：Long non-protein-coding RNAs (lncRNAs) are key regulators in gene expression. However, lncRNAs associated testis-specific expression remains functionally undetermined. Firstly, I have discovered candidates for testis-specific lncRNAs. The aim of this research is (1) To characterize the newly identify testis-specific lncRNAs in mouse and (2) to perform functional analysis of the testis-specific lncRNAs. As results, the antisense lncRNA 1700108J01Rik and long intergenic non-coding RNA 1700101022Rik were identified as testis-specific lncRNAs. These lncRNAs showed different stage-specific and cell-type specific expression pattern during mouse spermatogenesis. Next, I tried to do functional analysis of these lncRNAs by in vivo lentiviral-mediated transfection and target gene manipulation (still in under investigation).

研究分野：biology reproduction

キーワード：long non-coding RNAs　testis-specific lncRNA　spermatogenesis　testis

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９、ＣＫ－１９（共通）

１．研究開始当初の背景(background)

Long non-protein-coding RNAs (lncRNAs) play as key regulators in gene expression. LncRNAs (length > 200 nucleotides) show tissue- or cell type-specific expression patterns and they play important roles in biological processes such as cellular differentiation and tissue development (Fatica and Bozzoni, Nat Rev Genet, 15: 7-21, 2014). Spermatogenesis, a process of male germ cells differentiation, is important for transmission of genetic information and fertility of male production. LncRNAs are known to involved in regulation of spermatogenesis process (Akhade et al., Nucleic Acids Res 44: 387-401, 2016, Sun et al., PLoS One. 10, 8: e75750, 2013). There are few reports about testis-specific lncRNAs. LncRNAs are known to function as molecular signaling for significant functional biological process, molecular decoy at transcriptional level, guiding ribonucleoprotein for chromatin modification, and molecular scaffold that associated with epigenetic matters (Wang et al., Mol Cell 43: 904-914, 2011). At First, I have discovered testis-specific lncRNAs candidates, which exclusively express at specific stages of mouse spermatogenesis. However, there is little report about the functions of testis-specific lncRNAs.

２．研究の目的(purpose)

The purpose of this research is

(1) To characterize the newly identify testis-specific lncRNAs in mouse

The lncRNAs which are highly expressed in mouse testis by bioinformatics analysis were selected as testis-specific lncRNA candidates. After confirmation of expression level of selected lncRNA candidates in mouse testis, the expression pattern and cellular localization of these lncRNAs during spermatogenesis process was examined.

(2) Functional analysis novel testis-specific lncRNAs in mouse

In vivo functional analysis: examination of loss of function of testis-specific lncRNAs using viral-mediated shRNA gene delivery in mouse testis (OR) In vitro functional analysis: examination of loss of function or gain of function in gene-manipulated mouse male germ cell lines

３．研究の方法(research method)

(1) To characterize the newly identify testis-specific lncRNAs in mouse

- Bioinformatics analysis was previously performed to determine the candidates of testis-highly-specific lncRNAs in mouse using FANTOM5 mouse database.
- Reverse transcription quantitative polymerase chain reaction (real-time PCR) was conducted for confirmation of expression level of lncRNA candidates in different developmental stages of mouse testis compared to other adult organs.
- In situ hybridization (ISH) analyses of adult testis using target lncRNAs probes were

performed to detect the expression and cellular localization of testis-specific lncRNAs during mouse spermatogenesis.

(2) Functional analysis novel testis-specific lncRNAs in mouse

- In vivo target gene manipulation of mouse testis by viral-mediated microinjection of postnatal mouse testis. I performed both EGFP-tagged lentiviral-mediated and EGFP-tagged retro-viral mediated microinjection of retes testis area in postnatal day (PD) 8-10 (Please see **Fig.1**).
- Two months after microinjection, I performed immunohistochemistry (IHC) for anti-GFP antibody followed by DAPI to check the expression of transfection in target spermatogenetic cells in adult mice.

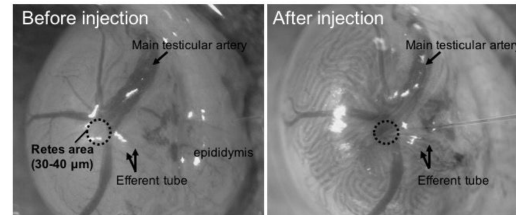

Fig. 1. GFP-tagged lentiviral vector microinjection into seminiferous tubules via retes area in PD 7 pup. After injection, trypan blue dye can be seen inside the seminiferous tubules.

- Design the target gene specific siRNAs and check the transfection efficiency of these siRNAs. The sequence of testis-specific lncRNAs (the antisense lncRNA *1700108J01Rik* and long intergenic non-coding RNA *1700101O22Rik*) were targeted by siRNA sequences designed using BLOCK-iT RNAi Designer (Invitrogen, CA, USA). The designated siRNAs were sequence-verified. The oligonucleotides were inserted into DsRed vector, allowing for determining transfection efficiency by fluorescence microscopy.
- In vitro analysis in COS-7 cells to check the transfection efficiency of EGFP-*1700108J01Rik* lncRNA, EGFP-*1700101O22Rik* lncRNA and their respective DsRed-siRNAs.

４. 研究成果(result)

(1) Characterization of testis-specific lncRNAs in mouse

According to the bioinformatic analyses and RT-PCR, *1700108J01Rik* lncRNA and *1700101O22Rik* lncRNA were found as testis-specific lncRNAs when compared to other adult organs (Please see **Fig. 2**). As results of ISH, both lncRNAs were positively-labelled in the spermatogenic germ cells inside the seminiferous tubules, but not in the

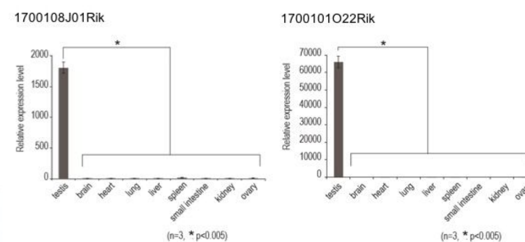

Fig. 2. Real-time PCR analysis showing that 1700108J01Rik and 1700101O22Rik are testis-specific lncRNAs when compared to other mouse adult organs.

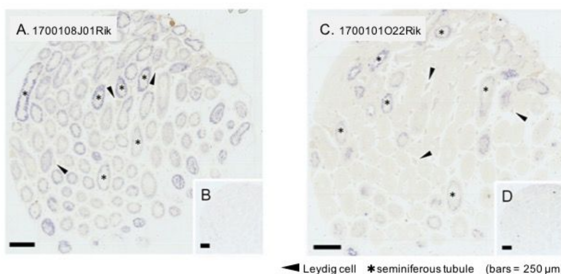

Fig. 3. ISH analysis of *1700108J01Rik* and *1700101O22Rik* in adult mouse testis.

interstitial cells (Please see **Fig. 3**). In Fig. 3, hybridization with anti-sense probe for (A) *1700108J01Rik* and (C) *1700101O22Rik* showing purple-color for positive signals. Hybridization with sense probe for either of (B) *1700108J01Rik* and (D) *1700101O22Rik* showed no special signal. Antisense lncRNA *1700108J01Rik* was detectable in seminiferous tubules at all stages (I to XII) of spermatogenesis, while *1700101O22Rik* lncRNA was expressed in stage VI to IX. Therefore, these two lncRNAs showed stage-specific expression pattern throughout different stages of spermatogenesis. *1700108J01Rik* was expressed in germ cells differentiating from stage VI pachytene spermatocytes to step 10 spermatids (Please see **Fig. 4**). *1700101O22Rik* was expressed exclusively in germ cells differentiating from step 6 to step 9 spermatids (Please see **Fig. 5**). In each stage of the cycle, the representative image of pseudo-colored signals (green) indicating J01Rik or O22Rik is shown on the left panel and the merged image of pseudo-colored signals (green) and DAPI-positive nuclei (blue) is shown on the right panel. Both of lncRNAs were mainly localized in the cytoplasm, but not in the nucleus of these germ cells. Therefore, these testis-specific lncRNAs have cell-type and stage-specific expression during mouse spermatogenesis. These findings indicate that these two testis-specific lncRNAs may involve specifically in male spermatogenesis functions such as germ cell differentiation or proliferation or apoptosis etc.

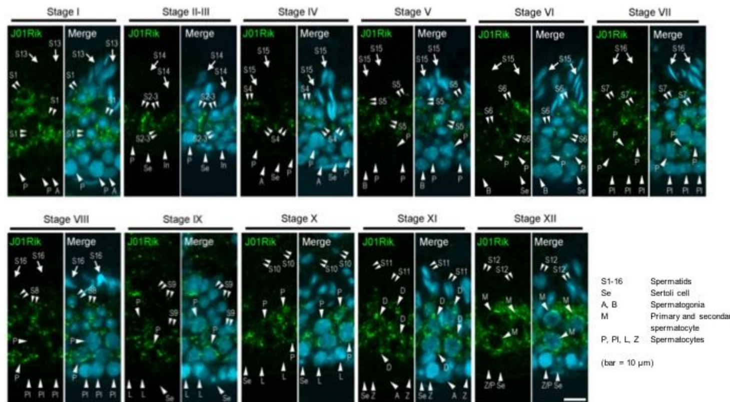

Fig. 4. Cellular localization of 1700108J01Rik (J01Rik) during the process of mouse spermatogenesis

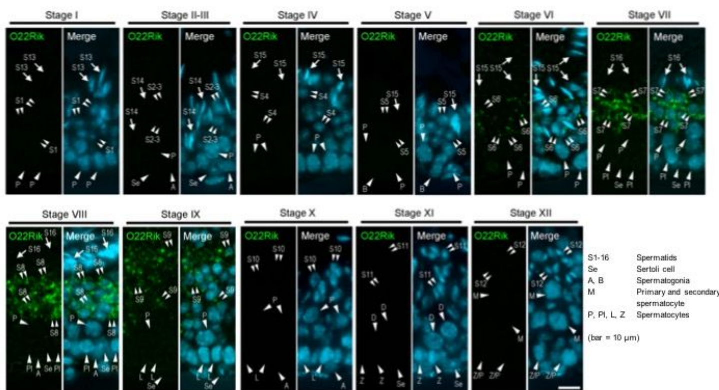

Fig. 5. Cellular localization of *1700101O22Rik* (O22Rik) during the process of mouse spermatogenesis

(2) Functional analysis novel testis-specific lncRNAs in mouse (in vivo analysis)

I performed viral-mediated in vivo microinjection of mouse testis at postnatal day 8-10 and check transfection expression by IHC under fluorescence microscopy on 2 months after microinjection. As results, lentiviral-mediated transfection showed more stable expression when compared to retrovirus. The expression of lentiviral transfection (anti-GFP; red color) was seen in the seminiferous tubules, but not in the interstitial cells (Please see **Fig. 6**). I checked the cell-type at different stages of spermatogenesis using DAPI-staining (DAPI-stained nuclei, blue

color). However, transfection was not mainly seen in the target spermatogenetic cells (either *1700108J01Rik* or *1700101O22Rik*-expressed cells). Sertoli cells and its cytoplasmic processes were mainly transfected (Please see **Fig. 7**). Even though EGFP-tagged lentiviral in vivo transfection was successful, I could not detect transfection on testis-specific lncRNA positive cell-type. Therefore, I tried to manipulate my primary research plan of in vivo analysis to in vitro functional analysis. At the same time, I will try to change the in vivo transfection methods to transfect the target cells.

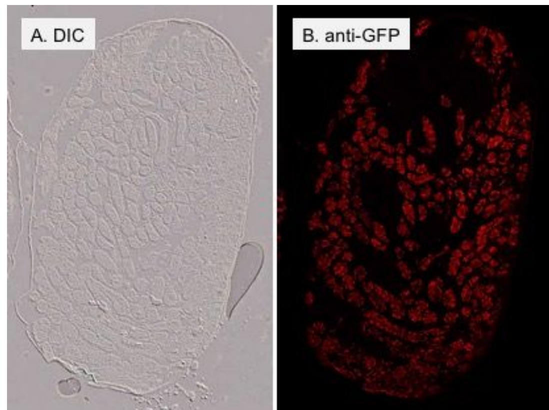

Fig. 6. EGFP-tagged lentiviral transfection in mouse testis: transfection was visualized by anti-GFP (red) inside the seminiferous tubules.

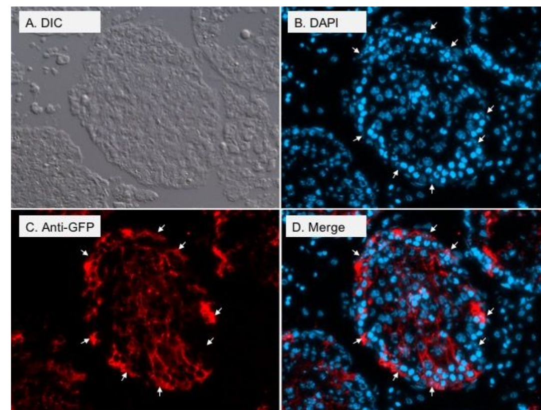

Fig. 7. Representative image of EGFP-tagged lentiviral transfection in a cross-section of seminiferous tubule. Lentiviral transfection (red color), DAPI-stained nuclei (blue color). Merge image showed that transfection was mainly seen in nuclei of Sertoli cells (shown by arrows) and its cytoplasmic processes.

５．主な発表論文等(main presentation paper)

〔雑誌論文〕（計 1 件）+ manuscript finished paper (**計 1 件)**

① Song X, **Kyi-Tha-Thu Chaw**, Takizawa T, Naing BT, Takizawa T.
1700108J01Rik and 1700101O22Rik are mouse testis-specific long non-coding RNAs. Histochem Cell Biol. 2018 May;149(5):517-527. doi: 10.1007/s00418-018-1642-4. Epub 2018 Feb 6. (https://www.ncbi.nlm.nih.gov/pubmed/29411102)

② **Kyi-Tha-Thu Chaw**, Banyar Than-Naing, Toshihiro Takizawa.
Localization of *H19* lncRNA and *miR-675-3p* in trophoblast cells during mouse placenta development. (manuscript draft preparation is ready to submit on journal of development biology.

〔学会発表〕（計 6 件）

① **マウス精巣における** *1700108J01Rik* **長鎖ノンコーディング** RNA **の発現解析**
(Expression of *1700108J01Rik* long non-coding RNA in the mouse testis)
チータートウー チョウ、宋 暁輝、瀧澤 敬美、瀧澤 俊広
日本医大・分子解剖
第 32 **回日本生殖免疫学会**（3rd Dec, 2017）
② **マウス脱落膜ナチュラルキラー細胞は** I **型グルコース輸送体**（Slc2a1）**を発現している**
(Decidual natural killer cells express glucose transporter type 1

(Slc2a1) during mouse pregnancy)
チータートウー　チョウ、 瀧澤　俊広
日本医大・分子解剖
第 25 **回日本胎盤学会学術集会** (24th Nov, 2017)
③ **マウス子宮ナチュラルキラー細胞に発現している** Slc2a1 **の組織化学的解析**
チータートウー　チョウ、 瀧澤　俊広
日本医大・分子解剖
第 123 **回日本解剖学会総会全国学術集会** (29th March 2018)
④ **マウス子宮** NK **細胞の** I **型グルコース輸送体（**Slc2a1**）は活性化されると細胞内顆粒から細胞表面にアップレギュレーションされる：組織化学的解析**
チータートウー　チョウ、 瀧澤　俊広
日本医大・分子解剖
第 70 **回産婦人科学会** (13th May 2018)
⑤ Histochemical analysis of Slc2a1 (glucose transporter type I) in uterine natural killer cells during mouse pregnancy
Chaw Kyi-Tha-Thu, Toshihiro Takizawa
Department of Molecular Medicine and Anatomy, Nippon Medical School, Tokyo, Japan
International Federation of Placenta Associations (IFPA) Tokyo, 2018 (22nd Sept, 2018)
⑥ FANTOM5 **データを用いたマウス精巣発生における長鎖ノンコーディング** RNA **の発現解析**
(Long non-coding RNA expression analysis during mouse testis development using the FANTOM5 data)
チータートウー　チョウ、 瀧澤　俊広
日本医大・分子解剖
第 33 **回日本生殖免疫学会** (24th Nov, 2018)

〔図書〕（計 0 件）

〔産業財産権〕
○出願状況（計 0 件）

○取得状況（計 0 件）

〔その他〕
ホームページ等

6．研究組織 **：なし**

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ローマ字氏名：

所属研究機関名：

部局名：

職名：
研究者番号（8桁）：

(2)研究協力者
研究協力者氏名：
ローマ字氏名：

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