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研究課題名（和文）A study of newly-identified testis-specific long non-coding RNAs in mouse spermatogenesis

研究課題名（英文）A study of newly-identified testis-specific long non-coding RNAs in mouse spermatogenesis

研究代表者
ＣＨＡＷ　ＫＹＩ．ＴＨＡ．ＴＨＵ（CHAW, KYI THA THU）

国際医療福祉大学・医学部・助教

研究者番号：００７９２４１７

交付決定額（研究期間全体）：（直接経費）　　3,200,000円

研究成果の概要（和文）：長い非ｺﾝﾃﾞｨﾝｸﾞ RNA（lncRNA）は、精子形成を含むさまざまな細胞ﾌﾟﾛｾｽにおける遺伝子発現の調節に関与している。しかし、精巣特異的 lncRNA の発現とその機能はまだ不明です。精巣特異的な lncRNA は以前に同定されており、ISH の結果は精子形成における J01Rik lncRNA の細胞特異的発現を示しました。本研究では、ﾏｳｽ精巣発育期における lncRNA の発現ﾊﾟﾀｰﾝを解析し、精巣特異的 lncRNA の役割を検討しました。また、環境汚染物質が生殖と発生に影響を与えるため、J01Rik 発現 GC2 細胞に対するﾋ素曝露の影響も調べました。

研究成果の学術的意義や社会的意義

This study provides new information about a functional testis-specific lncRNA and its regulatory network in spermatogenesis. Newly-identified J01Rik lncRNA was exclusively expressed in spermatocytes and it may be involved in regulation of cellular processes (cell cycle etc.)

研究成果の概要（英文）：Long non-conding RNAs (lncRNAs) are involved in the modulation of gene expression in various cellular processes including spermatogenesis. However, the expression of testis-specific lncRNAs and their functions are still unclear. The testis-exclusive lncRNAs had been identified previously and ISH results showed cell-specific expression of J01Rik lncRNA in spermatogenesis. In this study, I analyzed the expression patterns of lncRNAs during mouse testicular developmental periods and examined roles of testis-specific lncRNAs. I also examined the effects of arsenic exposure on J01Rik-expressed GC2 cells as environmental contaminants impact on reproduction and development.

研究分野：reproductive biology

キーワード：spermatogenesis　testis specific lncRNAs　arsenic

１．研究開始当初の背景

Most of the human genome encodes RNAs that do not code for proteins. They are called long non-coding RNAs (lncRNAs), which have more than 200 nucleotides in length. LncRNAs are reported as key regulatory transcripts in different biological processes (Krol et al., Nat Commun 6: 7305, 2015, Quinn and Chang, Nat Rev Genet 17: 47-62, 2016) including spermatogenesis (Luk et al., Reproduction 147: 131-141, 2014). Gene transcriptional regulation, post transcriptional regulation and epigenetic regulations are three main biological functions of lncRNAs. The location of lncRNAs in the cells provides clues to the molecular functions of novel lncRNAs. Some studies reported cytoplasmic lncRNAs with evidence of translational regulation (Van Heesch et al. 2014, Carlevaro-Fita et al. 2016). Therefore, localization of lncRNA expression is crucial in discovering the functions of novel lncRNAs. Cell-specific and tissue-specific expression profiles of lncRNAs have been greatly focused in various mammalian tissues. A few studies report about testis-specific lncRNAs in different mammalian species including mice (Jandura et al., Trends Genet 33: 665-676, 2017, Hong et al., BMC Genomics 19: 539, 2018). However, functional roles of many testis-specific lncRNAs are still unknown.

Spermatogenesis is a differentiation process of male germ cells (from spermatogonia to mature spermatozoa) in the seminiferous tubules of the testis. Spermatogenesis is a life time process and the regulators should exist in all stages of spermatogenesis. Profiling of lncRNAs in spermatogenesis showed that some lncRNAs are ubiquitously expressed in all germ cells suggesting to serve as housekeeping genes for entire process (Liang and Li et al., 2014) while certain lncRNAs may specifically express in different types of germ cells. Among the different types of lncRNAs such as intergenic lncRNAs, anti-sense lncRNAs, overlapped lncRNAs, bidirectional lncRNAs, intergenic and anti-sense lncRNAs contribute majority and significant changes in number of were seen in male germ cells (Liang and Li et al., 2014).

２．研究の目的

I previously published a paper about newly identified testis-specific lncRNAs (*J01Rik* etc.) which showed male germ-cells-specific expression patterns during adult mouse spermatogenesis (Song and Chaw Kyi-Tha-Thu et al., Histochem Cell Biol 149: 517-527, 2018, equally contributed first author, Grant-in-Aid for Young Scientists B, 2017-2018). In bioinformatics analysis, we found high expression of testis-specific lncRNAs, which was confirmed by real-time PCR. *In situ* hybridization (ISH) results revealed that those lncRNAs were significantly expressed in different stages of spermatogenesis. These lncRNAs were specifically expressed in male germ cells of meiotic stage during spermatogenesis. But, we still need to investigate not only the functional roles but also regulatory network of these novel testis-specific lncRNAs. Moreover, mammalian testis development and their biological functions are important for successful male fertilization. Therefore, the specific lncRNA expressions during the testis developmental stages should be identified for further research into the molecular mechanisms of lncRNAs function in mammalian testis development and spermatogenesis. The purpose of this study is to provide new information about a functional testis-specific lncRNA and its regulatory

network and also investigate the expression profiles of testis-specific lncRNAs during testis development.

３．研究の方法

(1) Functional analysis of *J01Rik* in mouse testis using *in vivo* lentiviral-mediated gene manipulation

- I confirmed the *J01Rik* expression in mouse testis and mouse spermatocyte cell lines using qPCR and *in situ* hybridization (ISH).
- I prepared shRNAs to specifically knockdown the target lncRNAs.
- I performed *in vivo* microinjection of mouse testis in 3-4-week-old mice at the timing of first wave of spermatogenesis (representative image, Figure 3A).
- When the injected mice became adult, the transfection efficiency of these shRNAs was checked in COS-7 cells by fluorescence microscopy (GFP) 2-3 week after microinjection and their knockdown efficiency was confirmed by real-time PCR.
- Analyzed the gross morphology changes of mouse testis and spermatogenesis in the seminiferous tubules.

(2) In silico analysis of testis-specific lncRNAs during the mouse testis development using FANTOM5 dataset

- I used functional annotation of the mammalian genome (FANTOM)-5 dataset to explore the differential expressions of lncRNAs in prenatal (embryonic) and postnatal testis.
- Cap analysis of gene expression (CAGE) dataset was used for gene expression profiling
- Normalized relative log expression (RLE) >10 tags per million (TPM) CAGE peaks for 1073 C57BL/6J *Mus musculus* samples including primary cells, tissues and organs. I focused analysis on mouse testis development from embryonic day 13 (E13) to adult (Figure 1).

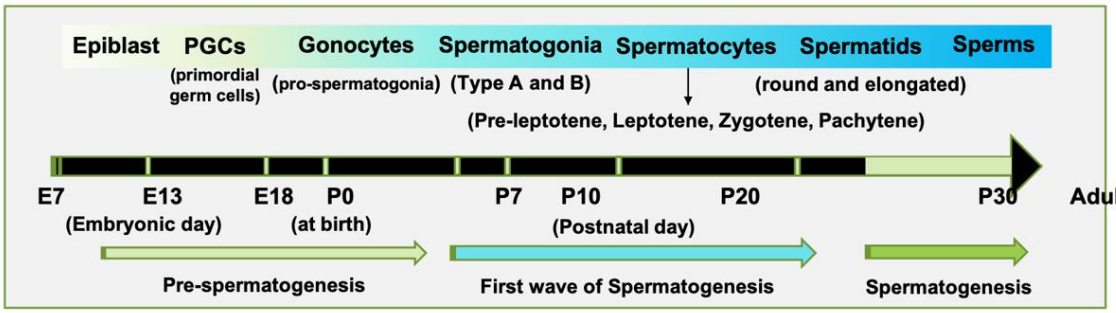

Figure 1. Different types of male germ cells in the spermatogenesis process at different developmental periods.

(3) Determine the impact of Arsenic exposure to the spermatogenic cells *in vitro* and Gene Set Enrichment Analysis (GSEA) Pathway analysis

- As *J01Rik* has a cell-specific expression in the spermatogenesis, particularly spermatocytes, I used GC-2 cells to confirm the gene expression by qPCR.
- The *J01Rik* expressed GC-2 cells were exposed with one of the environmental toxicants (0.1uM, 1uM and 5uM of Arsenic compound)

- The expression of *J01Rik* were examined after arsenic exposure by qPCR
- Genome-wide analysis by RNA-seq in the arsenic-exposed GC-2 cells were performed.

４．研究成果

(1) Functional analysis of *J01Rik* in mouse testis using *in vivo* lentiviral-mediated gene manipulation

Before the functional analysis, I confirmed that *J01Rik* is a new testis-specific lncRNA by qPCR and ISH (Figure 2). The qPCR data revealed that *J01Rik* was exclusively expressed in mouse testis and mouse spermatocyte cell line (GC-2). ISH results showed spermatogenic cell-specific expression pattern (i.e., pachytene spermatocytes and round spermatids) of *J01Rik* lncRNA. We observed that lentiviral-mediated knock down of *J01Rik* was efficient and qPCR data showed that *J01Rik* expression was downregulated in viral-infected COS-7 cells

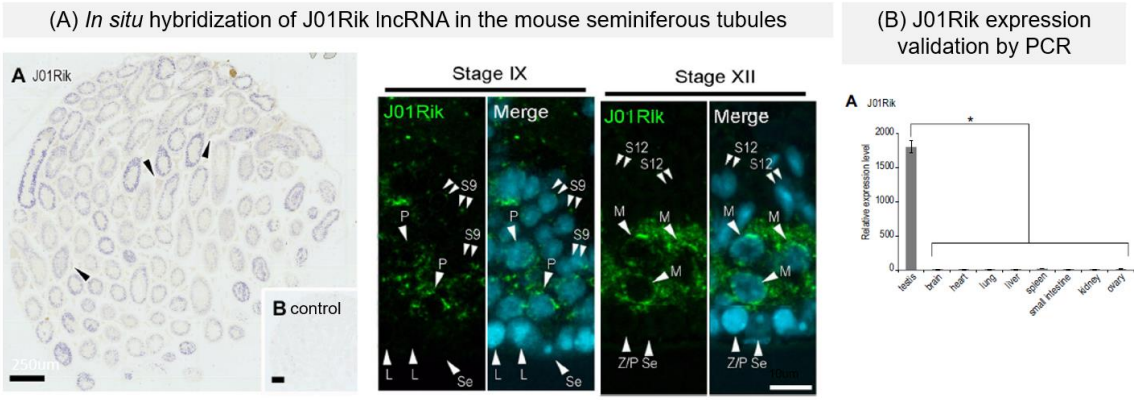

Figure 2. *J01Rik lncRNA was* confirmed as a testis-specific lncRNA, which may be involved in the mouse spermatogenesis. (A) *In situ J01Rik* lncRNA expression analysis in seminiferous tubules showed that *J01Rik* is specifically expressed in spermatocytes (pachytene (P), meiotically dividing spermatocytes (M) of spermatogenesis in the adult mouse testis. *J01Rik* expression (green). (B) qPCR data showing that *J01Rik* is a testis exclusive lncRNA. Published paper: Song & Kyi-Tha-Thu Chaw et al., Histochemistry and Cell Biology, 2018.

(Figure 3C). I chose shID4 and shID7 to knockdown the *J01Rik* expression for further analysis.

Fluorescence microscopy also revealed that viral transfected cells (red-colored in Figure 3B) were not only male germ cells, but also some Sertoli cells. The gene manipulated mice were sacrificed at 6 to 8-week-old and the weight and volume of the testis were measured. Both weight and volume of the *J01Rik* knockdown testis were significantly reduced when compared to vehicle

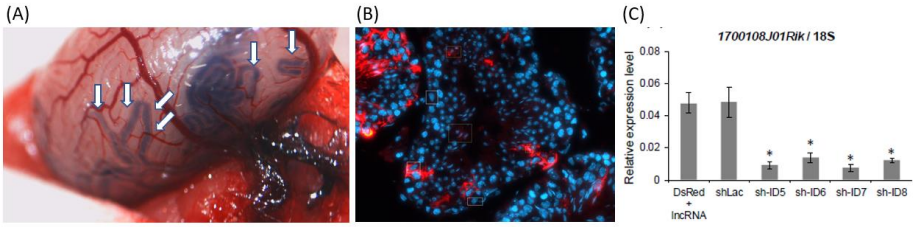

Figure 3. Representative images for in vivo functional analysis of *J01Rik* lncRNA. (A) lenti-viral mediated microinjection in the seminiferous tubules of mouse testis in 3 to 4-week-old mouse (white arrows show intratubular injection confirmed by trypan blue dye) and (B) cross section of seminiferous tubules of adult (6 to 8-week-old) mouse testis showing viral transfected male germ cells and sertoli cells (Red, viral infected cells, blue, dapi-stained nuclei). (C) qPCR analysis of expression of shRNAs in COS-7 cells showing that J01Rik expression was significantly reduced when compared to shLac control (*p <0.05).

control. Moreover, abnormal morphological changes of some spermatogenic cells in *J01Rik*

knockdown mouse testis were observed. Together, *J01Rik* is a functional testis-specific lncRNA which may involve in the mouse testis development.

(2) In silico analysis of testis-specific lncRNAs during the mouse testis development

I analyzed the top 10 to 100 highly expressed lncRNA at different stages of testis development such as pre-spermatogenesis (embryonic day (E) 13~18, early and late postnatal (P) periods P0~7 and P10~30. As results, long intergenic lncRNAs and anti-sense lncRNAs were the highest among lncRNA biotypes and Malat1 showed the highest expression in late embryo (E13~18) 75% of total lncRNA expression and also highest in the postnatal testis (P0~30) 79% in early and 26% in late postnatal testis. These data suggested that *Malat1* expression pattern is more accelerated in embryonic and neonatal stages (first wave of spermatogenesis) than in adult testis. But, *Malat1* was ubiquitously expressed in other tissues or organs (Figure 4). Therefore, *Malat1* may be involved in early testis developmental process, but it is not a testis exclusive lncRNA.

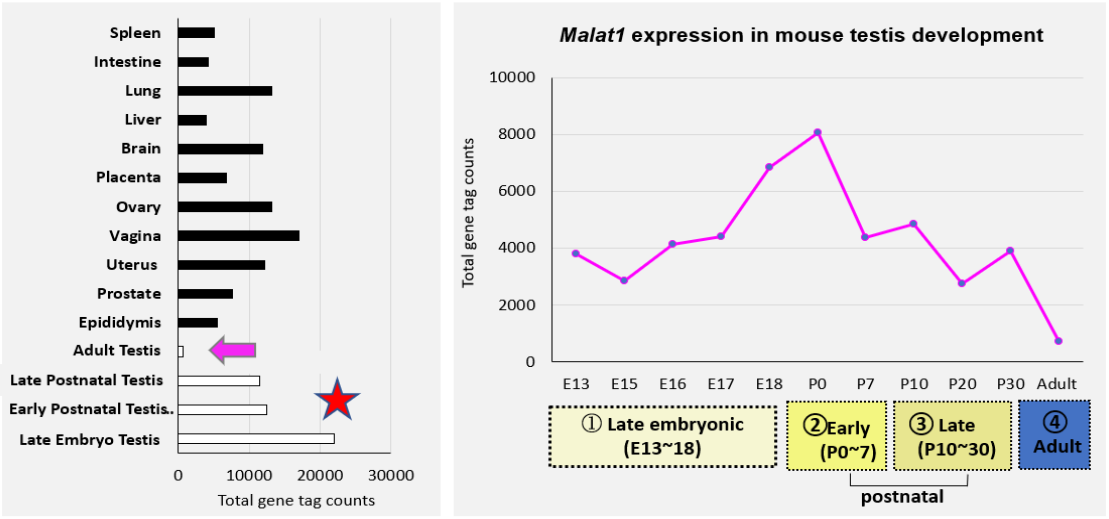

Figure 4. In silico data showing that *Malat1* expression levels in mouse testis development and other adult organs. Malat1 expression pattern is more accelerated in embryonic and neonatal stages (first wave of spermatogenesis) than in adult testis. But, Malat1 was ubiquitously expressed in other tissues or organs.

(3) Determine the impact of Arsenic exposure to the spermatogenic cells *in vitro* and Gene Set Enrichment Analysis (GSEA) Pathway analysis

Some studies reported that environmental contaminant like arsenic causes reproductive and developmental toxicity such as dysfunction of the testis. Additionally, we aimed to investigate the expression profiles of testis-specific lncRNAs during testicular developmental period and impact of arsenic exposure in GC-2 cells. Real-time PCR analysis showed that *J01Rik* was increased by arsenic (0.1uM 72Hr exposure) in the GC-2 cells. Furthermore, genome-wide analysis by RNA-seq in the arsenic-exposed GC-2 cells revealed several downregulated genes associated with regulation of cell cycle process and upregulated genes associated with regulation of apoptosis process and germ cell development.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計2件（うち招待講演　0件／うち国際学会　1件）

１．発表者名 CHAW KYI-THA-THU, Aya MISAWA, Tin-Tin WIN-SHWE, Takehiro SUZUKI
２．発表標題 Expression of Testis-specific Long Non-coding RNAs Related to Spermatogenesis and Impact of Environmental Contaminant Exposure
３．学会等名 Environmental Pollution and Health Impact on Future Generation in Asian Countries (EPHIF 2023)（国際学会）
４．発表年 2021年～2022年

１．発表者名 Chaw Kyi-Tha-Thu, Toshihiro Takizawa
２．発表標題 Long non-coding RNA expression analysis during mouse testis development using the FANTOM5 data.
３．学会等名 33rd Annual Meeting of Japan Society for Immunology and Reproduction
４．発表年 2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

I received a Best Oral Presenter Award 2023 for an outstanding presentation in Environmental Pollution and Health Impact on Future Generation in Asian Countries International Conference (EPHIF, 2023) on 14th March 2023 at Universiti Sultan Zainal Abidin (UniSZA), Kuala Terengganu, Malaysia.

The title of the presentation was "Expression of Testis-specific Long Non-coding RNAs Related to Spermatogenesis and Impact of Environmental Contaminant Exposure".

６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
研究協力者	瀧澤　　俊広 (Takizawa Toshihiro)	日本医科大学・分子解剖学・教授	

６．研究組織（つづき）

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
連携研究者	鈴木　武博 (Suzuki Takehiro) (60425494)	国立研究開発法人国立環境研究所・環境リスク・健康領域・主任研究員 (82101)	

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関