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研究課題名（和文）Placenta specific long-non-coding RNA analysis: a new molecular basis of miscarriage

研究課題名（英文）Placenta specific long-non-coding RNA analysis: a new molecular basis of miscarriage

研究代表者

ＢＡＮＹＡＲ　Ｔ．ＮＡＩＮＧ（Banyar, Than Naing）

日本医科大学・医学部・助教

研究者番号：６０７３２３８６

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研究成果の概要（和文）：MicroRNA miR-675-3pはlong noncoding RNAであるH19より産生され、マウス満期胎盤で高発現している。H19とmiR-675-3pの発現をISH法で解析した。結果、H19は胎盤関門を構成している胎児血管内皮細胞及び単核栄養膜細胞に発現していたが、その間に位置する合胞体栄養膜細胞には陰性であった。miR-675-3pは胎盤関門構成細胞全てに陽性であった。ISH解析により、miR-675-3pはH19発現細胞から分泌され、H19を発現していない近傍の栄養膜細胞に取り込まれることを示唆する新知見を見出した。

研究成果の概要（英文）：[Purpose] H19 is maternally imprinted long non-coding RNA and also serves as a precursor for miR-675. Even though both are highly expressed in the placenta, expression cell types are still not reported. This time, we analyzed expression of H19 and miR-675-3p in mice term placenta.[Methods] Total RNA extracted from B6D2F1 placenta (E7.5~E18.5) was used for expression of H19 and miR-675. Expression positive cells were identified by in situ hybridization (ISH) using E18.5 placenta.[Results] By ISH at E18.5 labyrinth, H19 was expressed in fetal vascular endothelial cells and mononuclear trophoblasts, but not in syncytio trophoblasts. But miR-675-3p was positive in all placental barrier constituent cells. In addition, miR-675-3p was also positive in H19 negative spongy trophoblasts in the junctional zone.[Conclusion] This is the first report that miR-675-3p produced from H19 expressing cells was secreted to nearby H19 negative cells and need further investigation for detail functions.

研究分野：　医歯薬学

キーワード：　産科学

１．研究開始当初の背景

In mammalian placenta, the **non-coding RNA** that does not encode a protein plays an important function such as regulation of gene expression has already reported. Recently, one of our department members has been discovered that microRNA (miR-517a-3p) of 20-25 bases in length is expressed specifically in placenta, and has a strong relationship with pregnancy immune (Kambe et al. Biol Reprod [in press] 2014).

In addition to short non-coding RNA, **long-non-coding RNAs (lncRNAs), with a length of 200 bases or more**, are class of genes involved in a variety of biological functions. As new lncRNAs are being discovered at a rapid pace (such as large-scale transcriptome analysis by next generation sequencing), the molecular mechanisms of lncRNAs are likely to be enriched and diversified. LncRNAs show cell type specific expression and respond to diverse stimuli, suggesting that their expression is under considerable transcriptional control (Wang et al. Mol Cell 43: 904–914, 2011). As such, lncRNAs can serve as molecular signals (**Fig. 1A**) because transcription of individual lncRNAs occurs at very specific time and place to integrate developmental cues, interpret cellular context, or respond to diverse stimuli (suppress or promote gene expression). In addition, lncRNA may be involved in genomic imprinting of the placenta (Wang et al. PNAS 110:10705 – 10710, 2013). However, there

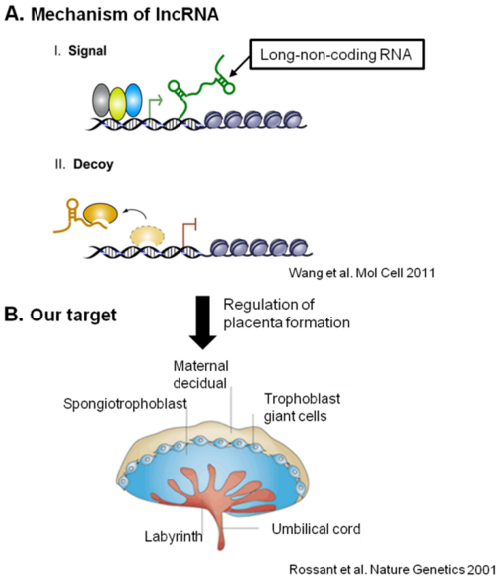


Fig. 1. Are long-non-coding RNAs involved in the formation of placenta?

are no reports about lncRNA expression in a placenta-specific manner (**Fig. 1**). Therefore, I would like to **identify placenta-specific lncRNAs by combining histochemistry and bioinformatics**. In addition, **I would like to prove that these lncRNAs are specifically expressed in the placenta by functional analysis using gene knock down and publish as the first report in the world**.

２．研究の目的

The main purpose of this study is "**to detect whether long-non-coding RNAs (lncRNA) are involved obviously in the formation of placenta, and to elucidate a new molecular basis of miscarriage**". I would like to (1) identify long-non-coding RNAs which are expressed specifically in the mouse placenta, and to elucidate the function of these genes in placenta by placenta-specific gene knockdown. (2) To analyze placenta-specific expressed lncRNAs that were identified in

the mouse and consider the involvement of these lincRNAs in human miscarriage such as unexplained early abortion cases which are not due to genetic abnormalities.

３．研究の方法

I have already found some candidate placenta-specific lncRNA as a preliminary analysis: I have selected the mouse placenta-specific candidate lncRNAs, and among them, I finally selected *H19* lncRNA which was already reported specifically expressed in placenta but very few reports about expression level and expression cell types at different developmental stages. We also studied *miR-675* which was synthesized from exon 1 of *H19* lncRNA.

3.1. Animals (E7.5 – 18.5)

Sample Collection

We received the approval from Nippon Medical School Hospital Ethics Committee for using animals in this experiment. The pregnant B6D2F1 mice (E7.5, E10.5, E13.5, E16.5 and E18.5) and adult mice (8 weeks of age), weighing 22±3g, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Mice were anesthetized with an intraperitoneal injection of 0.1 ml/10kg mixture of medetomidine (0.3 mg/kg), Midazolam (4 mg/kg) and Butorphanol (5 mg/kg), and extracted brain, heart, lung, liver, kidney, intestine, spleen, ovary and testis from adult mice and placenta from pregnant mice. We extracted total RNA from all extracted adult organs for real-time PCR and also prepared placenta paraffin samples at five different developmental stages (E7.5, E10.5, E13.5, E16.5 and E18.5) for in situ hybridization (ISH).

3.2. Tissue preparation

For ISH, mouse placenta samples at five different developmental stages were harvested, washed in cold PBS, and fixed with 4% paraformaldehyde/PBS at room temperature for 12 hours. Subsequent processing steps include to be dehydrated through an ethanol series and in order to uniform staining, pieces of sliced placenta were embedded in paraffin. We prepared 3μm paraffin sections and mounted on glass cover slips and allowed to dry at 42°C for 2 hours. Then, the cover slips were stored at 4°C before ISH.

3.3. Probe preparation

For preparation of *H19* probe, we ordered clone ID 1100001A04 (accession number AK003142) from DNAFORM (Yokohama, Kanagawa, Japan). We first extracted plasmid from clone after cloning using Miniprep kit (Qiagen, Hilden, Germany). Sense and antisense digoxigenin (DIG)-labeled riboprobes were constructed by linearizing the plasmid using restriction enzymes followed by in vitro transcription using T7 or T3 RNA polymerase (Roche Molecular Systems, Inc, Pleasanton, CA) in the presence of DIG-UTP (Roche).

For *H19* expression detection, we used all five different developmental stages (E7.5, E10.5, E13.5, E16.5 and E18.5) samples and all adult organs. SYBR Premix ExTaq II

was used for Real-time quantitative analysis using ABI7300 (Applied Biosystems) according to the manufacturer's protocol.

For absolute quantification, we used all five different developmental stages samples and all adult organs.

3.5. In situ hybridization

For *H19* ISH, the paraffin cover slips of all five different developmental stages samples were deparaffinized and then were treated with proteinase K (1ug/ml; Wako) at 37°C for 10 min in 24-well plate. After that the samples were pre-hybridized with hybridization buffer that contained 50% deionized formamide, 100mg/ml dextran sulfate, 4X sodium citrate, 0.1X Denhardt solution (Invitrogen), 0.5 mg/ ml herring sperm DNA (Thermo Fisher Scientific), 0.002M EDTA (Wako) at 70°C for 1 hour in a moist chamber and subsequently hybridized overnight in the same hybridization solution containing 200 ng/ml 3′-digoxigenin (DIG)-labeled probe at 70°C. Post-hybridization washes (37°C) were carried out three times in 1ml of 2X SSC (1 L 20X SSC contains 175.3 g NaCl and 88.2 g sodium citrate) each for 5 min and another three times in 1ml of 60% Formamide and 0.1XSSC each for 5 min. After washing, the sections were incubated with anti-Digoxigenin-alkaline phosphatase, Fab fragments, from sheep (1:200 dilution; Roche, Basel, Switzerland) at room temperature for 2 hours and visualized with nitroblue tetrazolium chloride (NBT; Roche) and 5-bromo-4-chloro-3′-indolyphosphatase p-toluidine salt (BCIP; Roche) at 37°C for 6 hours. After ISH, sections were counterstained with 4′, 6-diamidino2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR), and then mounted in CC/ mount. Control sections received the same treatment with sense probes.

For miR-675-3P ISH, the paraffin cover slips (only E18.5) were deparaffinized and then were treated with proteinase K (1ug/ml) at 37°C for 10 min in 24-well plate. After that the samples were pre-hybridized with microRNA ISH Buffer Set (FFPE) from Exiqon Life Sciences (Vedbaek, Denmark), at 60°C for 1 hour in a moist chamber and subsequently hybridized for 3 hours in the same hybridization solution. Post-hybridization washes (37°C) were carried out one time in 1ml of 5X SSC (1 L 20X SSC contains 175.3 g NaCl and 88.2 g sodium citrate) for 5 min followed by two times of 1ml each of 1X SSC and 0.2 X SSC at 60°C. After washing, the sections were incubated the same as H19 ISH. Control sections received the same treatment using Scramble-miR probe.

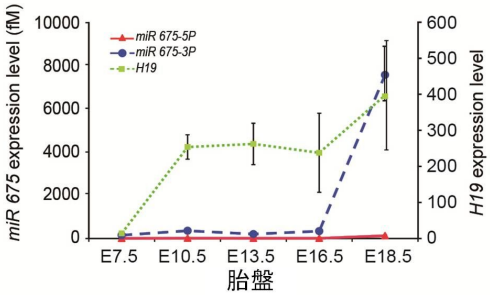
3.6. Morphological analysis

The sections were examined and then Bright-field, fluorescence, and differential interference contrast images were captured under Leica Application Suite X (DM6) microscope equipped with a DFC450C camera.

４．研究成果

Real-time PCR revealed that *H19* ncRNA was highly expressed in the placenta compared to E7.5, it was significantly upregulated at the later stages of placental development. We also analyzed *miR-675* expression by real-time PCR. *H19* showed highly expression in E10.5~E18.5 placenta. Expression of *miR-675* was lower at E10.5~E16.5 placenta, but was very high in E18.5 (Fig 2).

Fig 2. Real-time PCR of *H19* and *miR-675*



By ISH, *H19* was expressed in trophoblast giant cells (栄養膜巨細胞) and glycogen trophoblast cells (グリコーゲン栄養膜細胞) in the junctional zone (Fig 3). In labyrinth zone, *H19* was expressed in fetal vascular endothelial cells (胎児血管内皮細胞)and mononuclear trophoblasts (単核栄養膜細胞), but not in syncytio trophoblasts (合胞体栄養膜細胞). But *miR-675-3p* was positive in all placental barrier constituent cells. In addition, *miR-675-3p* was also positive in *H19* negative spongy trophoblasts (スポンジ 栄養膜細胞) in the junctional zone (Fig 3).

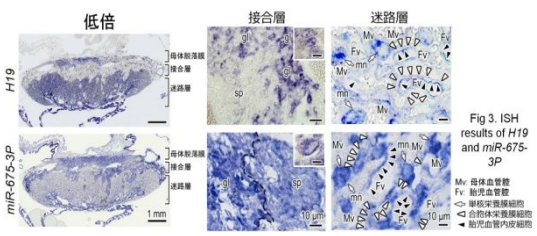


Fig 3. ISH results of *H19* and *miR-675-3P*

Mv: 母体血管腔
Fv: 胎児血管腔
◁▷ 単核栄養膜細胞
◁ 合胞体栄養膜細胞
◂ 胎児血管内皮細胞

We also confirmed that H19 was not expressed in the syncytio trophoblasts by ISH followed by immunohistochemistry using syncytio trophoblasts specific marker Slc2a1. Our results showed that *H19* was not expressed in the syncytio trophoblasts (Fig 4).

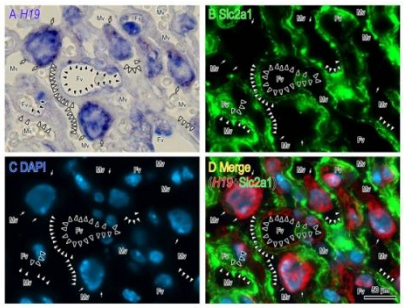


Fig 4. Results of *H19* and Slc2a1 double staining

Mv: 母体血管腔
Fv: 胎児血管腔
◁▷ 単核栄養膜細胞
◁ 合胞体栄養膜細胞
◂ 胎児血管内皮細胞

On the other hand, we also confirmed that *miR-675-3p* was expressed in syncytio trophoblasts by double staining *miR-675-3p* and Slc2a1. We could confirm that *miR-675-3p* was expressed in syncytio trophoblasts (Fig 5).

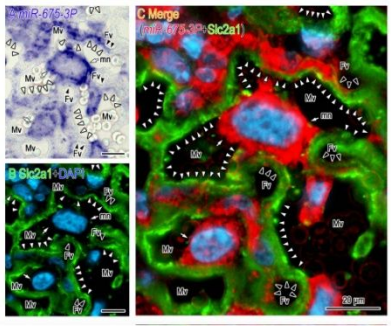


Fig 5. Results of *miR-675-3p* and Slc2a1 double staining

Mv: 母体血管腔
Fv: 胎児血管腔
◁▷ 単核栄養膜細胞
◁ 合胞体栄養膜細胞
◂ 胎児血管内皮細胞

Our finding showed that even though *miR-675-3p* was derived from exon 1 of *H19* lncRNA, it was expressed not only in all *H19* expressing cells but also in *H19* negative spongy trophoblasts (スポンジ 栄養膜細胞) in the junctional zone and syncytio trophoblasts (合胞体栄養膜細胞) in the labyrinth zone (Table 1).

Table 1. Summary of *H19* and *miR-675-3p* expression cells

胎齢 E18.5		*H19* lncRNA	*miR-675-3p*	
接合層	栄養膜巨細胞	+	+	
	スポンジ 栄養膜細胞	-	+	
	グリコーゲン栄養膜細胞	+	+	
迷路層	単核栄養膜細胞	+	+	胎盤関門構成細胞
	合胞体栄養膜細胞	-	+	
	胎児血管内皮細胞	+	+	

This is the first report about *H19* and *miR-675-3p* expression in placenta development cells. In addition, *miR-675-3p* was synthesized from *H19* but it was also expressed in the *H19* negative cells (spongy trophoblasts (スポンジ 栄養膜細胞) in the junctional zone and syncytio trophoblasts (合胞体栄養膜細胞)). This may be exported through exosome via parachrine function or via blood stream which we need further investigation.

５．主な発表論文等

（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　1　件）

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②Banyar Than Naing, Chaw Kyi Tha Thu, Toshihiro Takizawa「In situ hybridization **により明らかにされたマウス満期胎盤における** miR-675-3p **の傍分泌」 第** 122 **回日本解剖学会総会・全国学術集会**(2017.3)

③Chaw Kyi Tha Thu, Banyar Than Naing, Toshihiro Takizawa 「I **型グルコース輸送体**(Slc2al)**は子宮間膜側脱落膜リンパ系集合体に発現している」 第** 31 **回日本生殖免疫学会総会・学術集会**(2016.12)

④Chaw Kyi Tha Thu, Banyar Than Naing, Toshihiro Takizawa 「**マウス胎盤における** I **型グルコース輸送体の組織化学的解析」第** 24 **回日本胎盤学会学術集会・第** 34 **回日本絨毛性疾患研究会**(2016.11)

⑤Banyar Than Naing, Chaw Kyi Tha Thu, Toshihiro Takizawa 「**マウス胎盤迷路層における** H19 **は単核栄養膜細胞と胎児血管内皮に特異的に発現する」 第** 24 **回日本胎盤学会学術集会・第** 34 **回日本絨毛性疾患研究会**(2016.11)

⑥Banyar Than Naing, Toshihiro Takizawa “Expression analysis of H19 and miR-675 in mouse placental development” 68th Annual Congress of the Japan Society of Obstetrics and Gynecology(2016.4)

⑦ Banyar Than Naing, Xiaohui Song, Toshihiro Takizawa “Expression analysis of H19 non-coding RNA in the mouse placenta by in situ hybridization” **第** 121 **回日本解剖学会総会・全国学術集会**(2016.3)

⑧ Banyar Than Naing, Xiaohui Song, Toshihiro Takizawa “**マウス胎盤における** H19 **遺伝子の発現解析(第二報)” 第** 23 **回日本胎盤学会学術集会・第** 33 **回日本絨毛性疾患研究会**(2015.11)

６．研究組織

(1)**研究代表者**

Banyar T. Naing（Banyar T. Naing）

日本医科大学・医学部・助教

研究者番号：60732386