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Sex differences in placental structure and gene expression in ICR mice during embryonic development

Xinyi Man[1], Noriyoshi Usui[1,2,3,4,5]✉, Miyuki Doi[1,5] & Shoichi Shimada[1,3,4,5]

The placenta is a vital organ for fetal development, providing structural support and mediating both nutrient exchange and maternal–fetal immune interactions. However, there are many aspects of murine placental development, which are used as a model organism, that are not fully understood compared to humans. In this study, we examined sex differences in placental development in ICR mice, a commonly used outbred strain, by analyzing the junctional zone (JZ) and labyrinth (LAB) from embryonic day (E) 8.5 to E18.5. At E14.5, the JZ area and its proportion relative to the total placental area (JZ + LAB) were significantly larger in females than in males. Consistently, expression levels of JZ-associated genes were also higher in female placentas at this stage. In contrast, such sex differences in placental morphology and gene expression were not observed in C57BL/6J mice, suggesting that observed features are unique to the ICR strain. Collectively, our findings demonstrate a female-biased expansion of the JZ in ICR mice at mid-gestation and underscore the presence of strain-specific patterns in sex-dependent placental development.

Keywords Sex difference, Placental development, Decidua, Junctional zone, Labyrinth, Trophoblast

The placenta is a specialized organ crucial for mammalian reproduction, having evolved to support embryos within the maternal environment in viviparous species. This adaptation enhances fetal development and improves survival rates[1,2].

Placental development begins immediately after fertilization, with the decidua (DEC) (the modified uterine lining) undergoing significant transformations during pregnancy. The DEC is a critical region of the mouse placenta, playing an essential role in the maternal-fetal interface during pregnancy. It is derived predominantly from maternal tissue, undergoes significant changes during pregnancy, and plays an essential role in supporting fetal development. These changes create a supportive environment for implantation, providing structural support and promoting immune tolerance to prevent fetal rejection by the mother's immune system[3]. Trophoblastic cells from the blastocyst invade the uterine wall, establishing a critical connection with maternal tissues[4,5].

As pregnancy progresses, the placenta grows and matures, forming additional vascular structures to meet the fetus's increasing metabolic demands. The labyrinth (LAB) structure contains a complex network of maternal blood spaces and highly branched fetal capillaries within trophoblast layers, maximizing the surface area for efficient exchange of oxygen, nutrients, and waste products, while serving as a protective barrier against potentially harmful substances in maternal circulation[6]. The LAB also supports the development of specialized endothelial and trophoblastic cell types, ensuring proper fetal nourishment and protection[6]. The junctional zone (JZ), located between the DEC and LAB, plays a crucial role in endocrine function and serves as a source of energy through glycogen storage[7–9]. It contains specialized trophoblastic cells, such as spongiotrophoblasts and glycogen trophoblasts, which facilitate communication between maternal and fetal tissues and help regulate immune tolerance during pregnancy[5].

Despite structural and cellular differences, the mouse placenta shares key functional similarities with the human placenta. Both exhibit hemochorial organization, where syncytiotrophoblasts mediate nutrient and gas

[1]Department of Neuroscience and Cell Biology, Graduate School of Medicine, The University of Osaka, 2-2 Yamadaoka, Suita 565-0871, Osaka, Japan. [2]Omics Center, Center of Medical Innovation and Translational Research, Graduate School of Medicine, The University of Osaka, Suita 565-0871, Japan. [3]United Graduate School of Child Development, The University of Osaka, Suita 565- 0871, Japan. [4]Global Center for Medical Engineering and Informatics, The University of Osaka, Suita 565-0871, Japan. [5]Addiction Research Unit, Osaka Psychiatric Research Center, Osaka Psychiatric Medical Center, Osaka 541-8567, Japan. ✉email: usui@anat1.med.osaka-u.ac.jp

exchange. Mouse trophoblast stem cells resemble human cytotrophoblasts in terms of stemness and lineage potential. Invasive trophoblast subtypes, including spongiotrophoblasts, giant cells, and glycogen cells, parallel human extravillous trophoblasts in uterine anchoring and spiral artery remodeling. These conserved features support the utility of the mouse as a model for studying human placental development and related disorders[2,5,10]. Humans support longer gestation periods through extensive hormonal and nutrient supply and unique adaptations for maternal-fetal immune tolerance, while mice prioritize rapid growth and quick nutrient transfer, with a distinct immune profile that facilitates shorter-term immune interactions[11,12].

Mammalian placentas, including those of human and rodent, exhibit sex-specific characteristics that influence fetal growth and development[13]. Previous studies have found that fetal sex significantly influences gene expression in the early human placenta, with X/Y chromosome dosage compensation mechanisms likely contributing to the observed differences[14]. In rodents, male rat placentas show greater vascular development and surface area compared to females[15]. Sex differences in embryonic development are evident from early stages in both species. Male mouse embryos tend to grow faster during pre-implantation, potentially affecting implantation timing and subsequent placental development[16]. Female human embryos often exhibit greater resilience to adverse conditions, likely due to the presence of two X chromosomes, which enable compensatory gene expression[13,17].

Understanding these sex differences may have significant clinical implications, such as recognizing that male fetuses are at higher risk for complications like intrauterine growth restriction (IUGR), low birthweight (LBW), and preterm birth, which allows for targeted prenatal monitoring[2,13]. Epidemiological studies have reported that male are more prone to developing neurodevelopmental disorders like autism spectrum disorder (ASD)[18,19]while depression is more common in females[17]. Furthermore, distinct hormonal environments may influence the risks of conditions such as preeclampsia and gestational diabetes[20,21]. These findings underscore the need to incorporate sex as a biological variable in reproductive biology research, which is often overlooked in many studies.

While sex differences in placental development have been extensively studied in C57BL/6 mice, including comparative analyses with ICR mice at various stages, most of these studies have focused on pathological conditions or overall fetal and placental weight[22,23]. In contrast, few studies have systematically examined sex differences in distinct placental compartments—such as the areas of individual placental zone—under normal physiological conditions in ICR mice[24–26].

In the present study, we investigated sex differences across distinct placental regions in ICR mice under physiological conditions. Notably, we identified both histological and gene expression differences in the JZ at E14.5, indicating sex-biased development in this compartment.

Results

Sex difference in the developing placenta of ICR mice

To investigate sexual differences in fetal development, we analyzed the placentas of ICR mice. Placentas from E8.5 to E18.5 were Hematoxylin-Eosin (HE)-stained and morphologically divided into the DEC, JZ, and LAB (Fig. 1). Placental morphology underwent dynamic changes during development in both males and females, with no remarkable sex differences observed in overall morphology (Fig. 1).

Consequently, we quantified the area of each placenta across developmental stages. The placenta exhibited growth with gestational age, with no significant sex differences observed in the total area of the JZ and LAB between females and males from E12.5 to E18.5 (Fig. 2A, B). However, we found that the sex differences in the JZ area at E14.5, with males having smaller JZ area than females, but no changes were observed at other stages from E12.5 to E18.5 (Fig. 2C). Similarly, no differences in the LAB area were observed from E12.5 to E18.5 (Fig. 2D). Regarding the ratio of the JZ area to the total JZ and LAB areas, the JZ area ratio was significantly larger in females than in males at E14.5 (Fig. 2E).

These results indicate a sex difference in the JZ area in ICR mice, specifically at E14.5.

Expression of JZ-associated genes in the placenta of ICR mice

Next, we investigated whether the expression of JZ-associated genes was also increased in females, given that the JZ area was larger than in males. We found significant increases in the mRNA expression of *Tpbpa* (Fig. 3A) and *Psg17* (Fig. 3B) in the placenta of female ICR mice compared to males at E14.5. We also found trends toward increased mRNA expression of *Vegfa* (Fig. 3C) and *Prl3b1* (Fig. 3D) in the placenta of female ICR mice compared to males at E14.5. These results also indicate that a sex difference in the expression of JZ-associated genes between females and males in ICR mice at E14.5.

Strain and sex difference in the placenta between ICR and C57BL/6J mice

Finally, we analyzed whether the sex difference in the JZ area observed in ICR mice at E14.5 was also present in C57BL/6J mice at the same stage. No sex differences were observed not only in the JZ area but also the total area of the JZ and LAB, LAB area, and the ratio of the JZ area to the total JZ and LAB areas in C57BL/6J mice at E14.5 (Fig. 4A-D). These results indicate that the sex difference in the JZ area at E14.5 is a specific phenotype in ICR mice.

Discussion

In this study, we identified sex and strain differences in placental development using ICR and C57BL/6J mice. At E14.5, the JZ area and the ratio of the JZ area to the total JZ and LAB areas were significantly larger in female ICR mice. Consistent with the histological findings, we also found increased expression of JZ-associated genes in the placenta of female ICR mice compared to males at E14.5. Furthermore, we examined sex differences

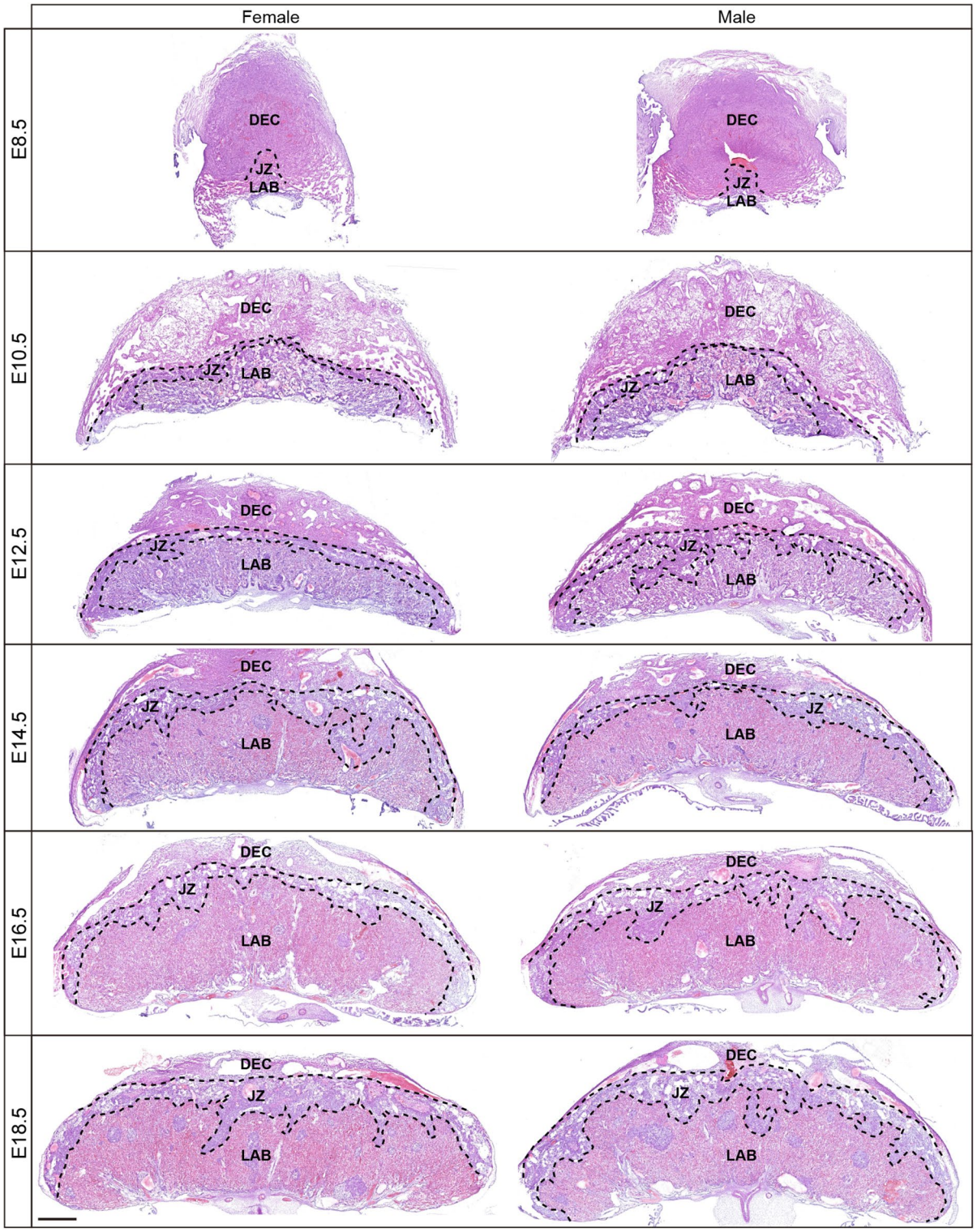

Fig. 1. Placental development between female and male ICR mice. Representative images of developing placenta from embryonic day (E) 8.5 to near birth at E18.5. Dotted lines indicate the borders between the maternal decidua (DEC), junctional zone (JZ), and labyrinth (LAB). Scale bar: 500 μm.

in placental regions in C57BL/6J mice at the same embryonic stage using histological analysis and found no apparent differences. These findings suggest that the histological sex difference observed in the JZ at E14.5 may be specific to ICR mice.

Sex differences in ICR mice, an outbred strain widely used worldwide, have been observed in various studies. In the animal models to human disorders such as ASD and cognitive impairment, ICR mice exhibit sex-specific

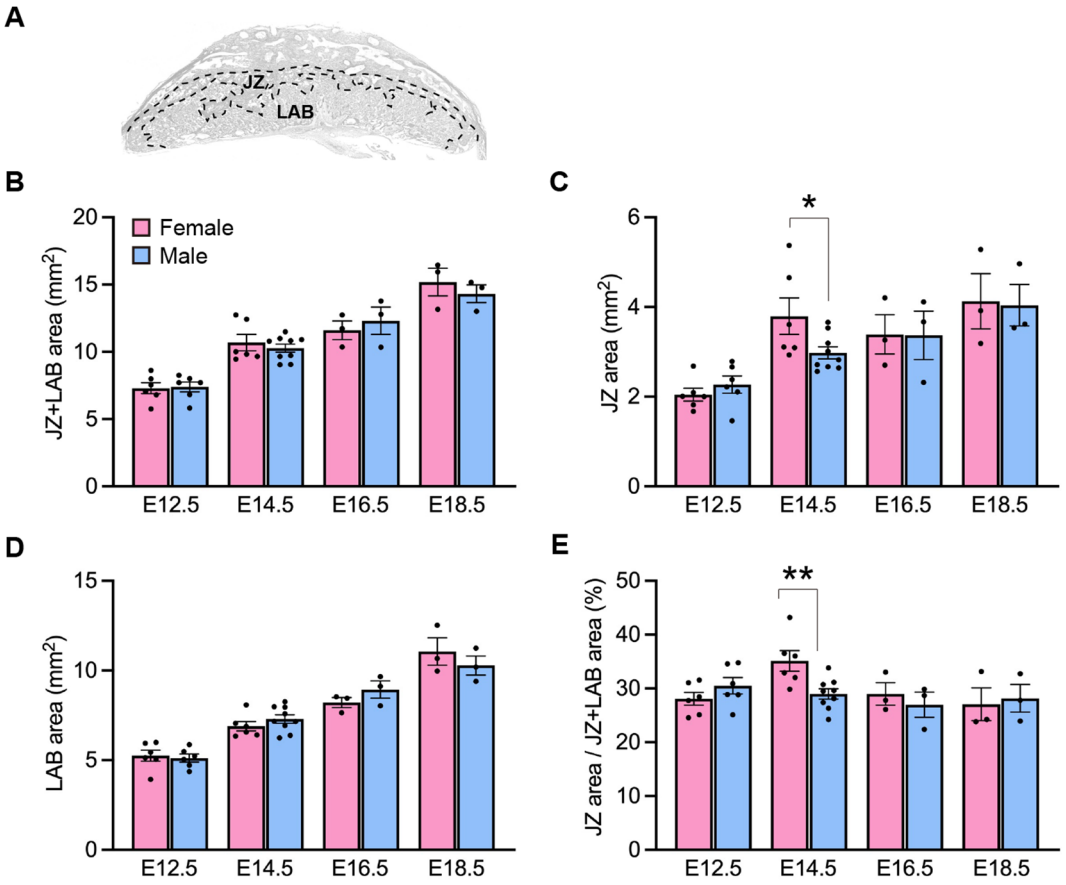

Fig. 2. Sex difference in the placental size between female and male ICR mice during embryonic stages.**A** Representative image of placental regions for quantification. **B-E** Sex differences in the total area of the JZ and LAB (**B**), JZ (**C**), LAB (**D**), the ratio of the JZ area to the total JZ and LAB areas (**E**) of ICR mice at E12.5 to E18.5. Sex difference was observed in the JZ at E14.5 (E12.5: $n = 6$ placentas from 2 pregnant mice; sex ratios: female: male = 6:8 and 10:3; E14.5: $n = 6$–9 placentas from 2 pregnant mice; sex ratios: 7:5 and 8:7; E16.5: $n = 3$ placentas from 1 pregnant mouse; sex ratio: 8:4; E18.5: $n = 3$ placentas from 1 pregnant mouse; sex ratio: 7:5). Data are presented as means (± SEM), $^{**}P < 0.01$, $^{*}P < 0.05$, unpaired *t*-test between sex differences in same stages.

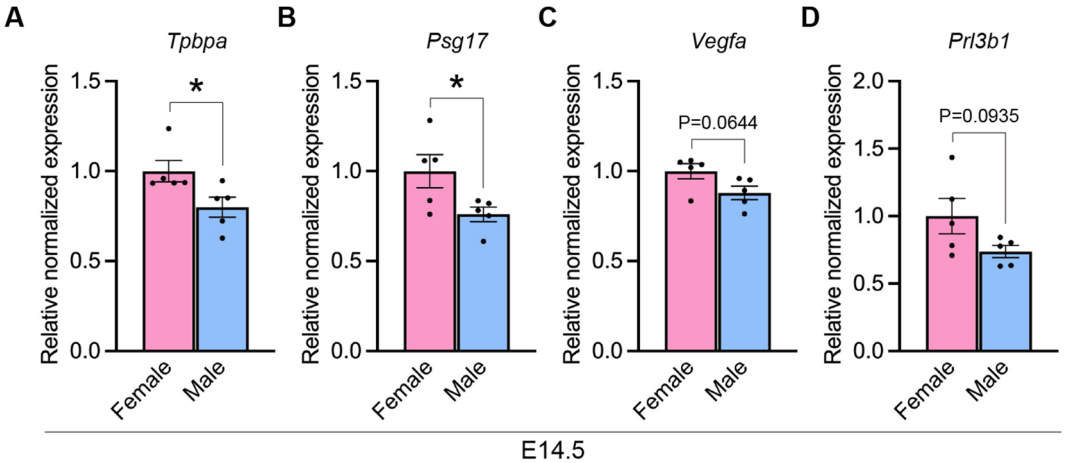

Fig. 3. Sex difference in JZ-associated gene expression in ICR mice. JZ-associated gene expressions in the placenta of ICR mice at E14.5. Higher expression levels of *Tpbpa* (**A**), *Psg17* (**B**), *Vegfa* (**C**), *and Prl3b1* (**D**) were observed in the placenta of female ICR mice compared to males at E14.5 ($n = 5$ placentas from 2 pregnant mice; sex ratios: female: male = 5:7 and 7:3). Data are presented as means (± SEM), $^{*}P < 0.05$, unpaired *t*-test.

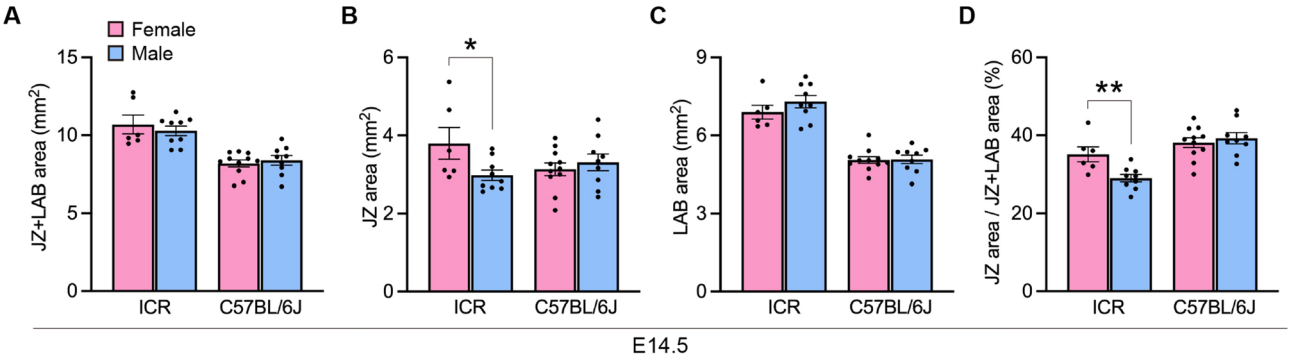

Fig. 4. Sex difference in the placental size in ICR and C57BL/6J mice.**A-D** Sex differences in the total area of the JZ and LAB (**A**), JZ (**B**), LAB (**C**), and the ratio of the JZ area to the total JZ and LAB areas (**D**) in ICR mice at E14.5 ($n = 6–9$ placentas from 2 pregnant mice; sex ratios: female: male = 7:5 and 8:7) and C57BL/6J ($n = 9–11$ placentas from 3 pregnant mice; sex ratios: 4:4, 4:2, and 4:3). Data are presented as means (± SEM), $^{**}P < 0.01$, $^{*}P < 0.05$, unpaired *t*-test.

behavioral phenotypes[27,28]. Male ICR embryos display different growth rates compared to females; at E13.5, male embryos are heavier than females, which may correlate with significantly higher mean spongiotrophoblast to labyrinth ratios in males at this stage[16]. At E14.5 and E18.5, male placentas of ICR mice are also heavier than those of females[22]. In this study, we also found that the ratio of the JZ area to the total JZ and LAB areas was significantly larger in females of ICR mice at E14.5, providing further evidence for inherent sex differences.

Moreover, understanding the sex differences in C57BL/6J mice, the most commonly used inbred mouse strain worldwide, is also important for interpreting experimental data[22,29,30]. Previous study has reported that male embryos in C57BL/6J mice tend to develop more rapidly than females, often exhibiting slightly larger body sizes at E14.5 and E18.5 of gestation[22]. The other study has also reported the differences in maternal weight (E0.5 to E18.5), embryo weight (E10.5 to E18.5), and placental weight (E10.5 to E18.5) between C57BL/6 and ICR mice during embryonic development[26]. The placenta is known to undergo hypertrophic growth as development progresses. It is in a pre-hypertrophic state during midgestation (E0.5–E16.5), but transitions to a hypertrophic phase during the end of gestation (E16.5–birth)[22]. Sex-dependent differences in the pre-hypertrophic (E14.5) and hypertrophic (E18.5) placentas of these two strains have also been reported[22]. A study has also reported that the placental weight of ICR mice is slightly larger than that of C57BL/6 mice at E14.5[26]. Similarly, we observed the differences in the total area of the JZ and LAB, LAB area, and the ratio of the JZ area to the total JZ and LAB areas between ICR and C57BL/6J mice at E14.5 (data not shown). However, we could not observe the differences in the placental phenotypes between male and female within C57BL/6J mice. Together, these findings suggest that the specific sex differences in the JZ area of ICR mice at E14.5 significantly influence sex differences in embryogenesis in this strain.

In many mammals, including humans, placental size is closely correlated with fetal size and birth weight. Placenta is a crucial organ for fetal development, playing a key role in supplying oxygen, vitamins, lipids, minerals, amino acids, glucose, and other essential nutrients to the fetus[31]. It also acts as a barrier, protecting the fetus from toxic substances[32] Larger placentas generally provide more resources to the developing embryos, often resulting in higher birth weights[16,33]. However, this correlation can be influenced by factors such as gestational length, maternal health, and species-specific adaptations.

The mouse placenta consists of three major layers: the DEC, JZ, and LAB, each with distinct functions. The DEC, composed of stromal cells, serves as a physical barrier[32]. The JZ, made up of spongiotrophoblasts and glycogen cells, synthesizes and stores glycogen as a nutrient source for the embryos[2,34–36]. The glycogen cells provide glucose to the embryos[32,37]. Our data indicate that sex differences begin to emerge during placental development, which may also affect embryo weight at earlier developmental stages.

There are several types of trophoblastic cells in the JZ, each with distinct functions in the development and maintenance of placental structure and function[2,34–36]. Spongiotrophoblasts are found deeper in the placenta, providing structural support and helping maintain a suitable environment for fetal growth[34,35]. Glycogen trophoblasts—originating from spongiotrophoblasts—are characterized by glycogen accumulation and have the capacity to migrate into the maternal decidua, where they may contribute to spiral artery remodeling. Spiral artery-invading perivascular interstitial trophoblasts and endovascular trophoblasts remodel the maternal spiral arteries, increasing blood flow to the placenta, which supports fetal growth[5,35,36].

The expression of various genes contributes to the trophoblastic cell development and differentiation[5,38–40]. Among these, we observed increased expression of JZ-associated genes, including *Tpbpa* and *Psg17*, as well as trends toward increased *Vegfa* and *Prl3b1* in the placenta of female ICR mice compared to males at E14.5. *Tpbpa* is a marker for specific trophoblast populations such as spongiotrophoblasts and glycogen trophoblasts in the placenta[40]playing a role in placental development, nutrient storage, and maternal-fetal interactions[5,41]. *Psg17* is a member of the pregnancy-specific glycoprotein (PSG) family, which belongs to the immunoglobulin superfamily, and is expressed in the spongiotrophoblast of the placenta during pregnancy[39,42]. It plays a role in immune tolerance, vascular development, and anti-inflammatory processes, thereby supporting a successful pregnancy[39,42]. PSG family including PSG17 also promotes angiogenesis by stimulating the production of pro-

angiogenic factors such as VEGF[43,44]. *Vegfa* is a growth factor that regulates placental blood vessel development, a process known as angiogenesis[45]. Proper vascularization of the placenta is essential for ensuring adequate nutrient and oxygen transfer from the mother to the fetus. *Prl3b1*, a member of the prolactin-related protein family, is expressed in the placenta, playing a role in supporting trophoblast function, immune tolerance, and placental development[36,40]. *Prl3b1* promotes maternal β-cell proliferation and insulin production during mid to late gestation and is expressed in a nutrient- and zone-specific manner in the placenta. Its expression in the JZ is responsive to maternal nutritional status, suggesting a potential role in directly modulating maternal physiology[46–48]. Our results raise the possibility that female placentas may enhance endocrine functions, such as nutrient transfer, immune tolerance, and maternal metabolic adaptation, potentially allocating more resources toward supporting both fetal development and maternal homeostasis during pregnancy.

The development of the JZ is crucial for supporting fetal growth and maintaining pregnancy health. Abnormalities in this region have been associated with complications such as IUGR and may influence the risk of neurodevelopmental disorders[34]. Male embryos, due to their higher growth demands, may be more vulnerable to placental insufficiency[13]. Impaired function of the JZ can affect nutrient and oxygen delivery, both essential for brain development, and is a known risk factor for LBW. For example, prenatal methamphetamine exposure has been shown to reduce glycogen-positive areas in the JZ area, leading to LBW[49]. LBW has been identified as one of several potential risk factors for ASD[50].

While the current study did not directly investigate ASD-related outcomes, our findings raise the possibility that early sex differences in placental development—particularly in the JZ—could modulate susceptibility to later neurodevelopmental outcomes. Notably, sex-specific placental and brain vulnerabilities have been reported in mouse models of maternal immune activation, in which male offspring often exhibit ASD-like behaviors[18,19,23,50–53]. These observations support the need for future studies to explore how early placental development may interact with environmental factors to influence long-term brain function in a sex-dependent manner.

Taken together, these findings suggest that the smaller JZ observed in male fetuses at E14.5—a critical period for placental and fetal development in mice—may contribute to sex-specific susceptibility to the prenatal environment during development. Our study represents an initial step toward characterizing sex-biased placental development under normal physiological conditions and provide a useful reference for future investigations in pathological contexts. Moreover, it may serve as a foundation for exploring how fetal sex interacts with placental structure to influence susceptibility to neurodevelopmental disorders under disease conditions. We acknowledge that placental weight, as well as maternal and fetal phenotypic characteristics, were not assessed in the present study, thereby limiting the scope of the available data. Furthermore, the use of placentas derived from a single dam in some analyses represents a methodological limitation that may affect the generalizability of our findings.

In conclusion, the JZ is larger in female than in male ICR mice at E14.5, accompanied by sex differences in the expression of JZ-associated genes. These findings raise the possibility that functional sex differences in the JZ could be one of several factors influencing sex-biased fetal growth trajectories.

Methods

Mice

ICR and C57BL/6J mice (Japan SLC Inc., Shizuoka, Japan) were used. Mice were housed (143 × 293 × 148 mm cages) under a 12-hour light-dark cycle with free access to food and water. Male and female mice were separately housed and paired at 16:00, then kept together overnight. The following morning at 9:00, vaginal plugs were checked to confirm mating. The pairs were then separated to prevent further mating and ensure accurate designation of E0.5. At the designated gestational day, mice were euthanized using an overdose of isoflurane followed by cervical dislocation, in compliance with ethical guidelines. In this study, ICR dams typically carried 11–15 fetuses per litter, and C57BL/6J dams carried 6–8 fetuses per litter. After embedding placentas, all measurements were performed by an investigator blinded to fetal sex and treatment group to eliminate potential bias related to sex and variations in fetal and placental weight. Sex genotyping was performed using the following primers: for *mSly-Xlr*, *F-5'*-GATGATTTGAGTGGAAATGTGAGGTA-*3'*, *R-5'*-CTTATGTTTATAGGCATGCA CCATGTA-*3'*.

HE stain

HE stain was performed as described previously[49]. Mouse placentas were fixed in 4% PFA overnight at 4 °C, cryoprotected in 30% sucrose, and embedded in Tissue-Tek O.C.T. Compound (#4583, Sakura Finetek Japan Co.,Ltd., Osaka, Japan). Representative mid-sagittal cryosections (20 µm) from the central placenta were stained with Hematoxylin (#131–09665; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 1% Eosin Y solution (#051-06515; FUJIFILM Wako Pure Chemical Corporation), dehydrated, and mounted with Permount (#SP15-100; Fisher Scientific, Pittsburgh, PA, USA). Images were captured with a BZ-X700 fluorescence microscope (KEYENCE, Osaka, Japan). The DEC, JZ, and LAB were manually delineated in each image using KEYENCE analysis software based on established morphological criteria.

qPCR

qPCR was performed as described previously[52]. Total RNA was extracted from mouse placenta at E14.5 using the miRNeasy Mini Kit (#217004; Qiagen, Hilden, Germany) following the manufacturer's instructions. Prior to the synthesis of single-stranded cDNA, genomic DNA contamination was removed from the total RNA using DNase I (#18068015; Thermo Fisher Scientific, Waltham, MA, USA). Single-stranded cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (#18080400; Thermo Fisher Scientific) and subsequently amplified by PCR. qRT-PCR was performed with PowerUp SYBR Green Master Mix (#A25742; Thermo Fisher Scientific) on a QuantStudio 7 Flex system under cycling conditions of 50 °C for 2 min, 95 °C for 2 min,

followed by 40 cycles of 95 °C for 1 sec and 60 °C for 30 sec. Each biological sample had four technical replicates, normalized to 18S rRNA. Data were analyzed using the ΔΔCq method with QuantStudio Software v1.7.2. The following primers were used: *18S rRNA*, F-5′-GAGGGAGCCTGAGAAACGG-3′, R-5′-GTCGGGAGTGGGTAATTTGC-3′; *mTpbpa*, F-5'-TGAAGAGCTGAACCACTGGA-3', R-5'-ACTCCCAGGCATAGGATGAC-3'; *mPsg17*, F-5'-CAGCAATATGGGAGTTGAAACA-3', R-5'-CTGTGCTGTCTGTGGCTTTT-3'; *mVegfa*, F-5'-CAGGCTGCTGTAACGATGAA-3', R-5'-GCATTCACATCTGCTGTGCT-3'; *mPrl3b1*, F-5'-TGTCAAGAACAAAGGAGTTGGA-3', R-5'-GACTGCAAATCTGACCATGC-3'.

Statistical analysis

Data are shown as means ± standard error of the mean (SEM) from independent experiments. Statistical analyses (F-test and unpaired *t*-test) were conducted using Prism 9. Significance levels are indicated as $^{**}P < 0.01$, $^{*}P < 0.05$, with $P < 0.05$ considered significant.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Xinyi Man: Validation, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. Noriyoshi Usui: Conceptualization, Methodology, Validation, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. Miyuki Doi: Validation, Investigation, Writing - Review & Editing, Visualization, Funding acquisition. Shoichi Shimada: Supervision.

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Declarations

Competing interests

The authors declare no competing interests.

Approval for animal experiments

All animal experiments were approved by The University of Osaka Animal Research Committee (#27 – 010), and conducted in accordance with relevant guidelines and regulations including ARRIVE guidelines.

Additional information

Correspondence and requests for materials should be addressed to N.U.

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