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—Original Article—

Impaired female fertility in tubulointerstitial antigen-like 1-deficient mice

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Abstract. Tubulointerstitial nephritis antigen-like 1 (Tinagl1, also known as adrenocortical zonation factor 1 [AZ-1] or lipocalin 7) is a matricellular protein. Previously, we demonstrated that Tinagl1 expression was restricted to extraembryonic regions during the postimplantation period and detected marked expression in mouse Reichert's membranes. In uteri, Tinagl1 is markedly expressed in the decidual endometrium during the postimplantation period, suggesting that it plays a physical and physiological role in embryo development and/or decidualization of the uterine endometrium during pregnancy. In the present study, in order to determine the role of Tinagl1 during embryonic development and pregnancy, we generated *Tinagl1*-deficient mice. Although *Tinagl1*$^{-/-}$ embryos were not lethal during development to term, homologous matings of *Tinagl1*$^{-/-}$ females and *Tinagl1*$^{-/-}$ males showed impaired fertility during pregnancy, including failure to carry pregnancy to term and perinatal lethality. To examine ovarian function, ovulation was induced with equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG); the number of ovulated oocytes did not differ between *Tinagl1*$^{-/-}$ and *Tinagl1*$^{flox/flox}$. *In vitro* fertilization followed by embryo culture also demonstrated the normal developmental potential of *Tinagl1*-null embryos during the preimplantation period. Our results demonstrate that Tinagl1 deficiency affects female mice and results in subfertility phenotypes, and they suggest that although the potential of *Tinagl1*$^{-/-}$ oocytes is normal, Tinagl1 is related to fertility in adult females but is not essential for either fertilization or preimplantation development *in vitro*.
Key words: Mouse, Pregnancy, Subfertility, Tubulointerstitial nephritis antigen-like 1 (Tinagl1)

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In mammalian reproduction, cross talk between the blastocyst and the uterine luminal epithelium is essential for the implantation process [1, 2]. Synchronized development of the embryo and the differentiation of the uterine endometrial cells to the receptive state are necessary for completion of this step. Uterine receptivity for implantation that supports blastocyst growth, attachment and the subsequent events of implantation is time limited [3–8]. The trophectoderm of implantation-competent blastocysts alters its functional programming *via* changes in cell surface molecules. The invasive trophoblasts of mouse blastocysts adhere, spread and migrate on extracellular matrix (ECM) substrates [9–12] and penetrate three-dimensional ECM structures [13]. The proliferation and differentiation of uterine endometrial cells are also crucial steps during peri-implantation. Many factors such as the ECM, adhesion molecules, lipid mediators and transcription factors are involved in this process; however, elucidation of the underlying molecular pathways has been hindered by their intricacy.

Extracellular proteins that do not contribute directly to the formation of structural elements in vertebrates but serve to modulate cell-matrix interactions and cell function are categorized as matricellular proteins [14]. The mouse tubulointerstitial nephritis antigen-like 1 (Tinagl1, also known as adrenocortical zonation factor 1 [AZ-1] or lipocalin 7) has been cloned from mouse adrenocortical cells and is known to be closely linked with zonal differentiation of adrenocortical cells [15]. The secretory protein Tinagl1 is a matricellular protein that interacts with both structural matrix proteins and cell surface receptors [16]. Furthermore, Tinagl1 has been identified as a component of the basal lamina based on its co-localization and binding ability with laminin-1 and collagens [16].

Our previous study revealed that Tinagl1 is associated with peri-implantation development in mouse embryos [17, 18]. During the preimplantation period, the expression of both *Tinagl1* mRNA

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and Tinagl1 protein increased just prior to implantation. In blastocysts, Tinagl1 expression was localized to the trophectoderm. Using a progesterone-treated delayed-implantation model, Tinagl1 was found to be upregulated in implantation-induced blastocysts following estrogen treatment [17]. During the postimplantation period, Tinagl1 expression was restricted to extraembryonic regions. Significant Tingal1 expression was detected in Reichert's membranes on embryonic days 6.5 (E6.5) and E7.5 [17]. Immunoprecipitation analysis determined that Tinagl1 binds with laminin-1 in embryos on E7.5 [17]. These results demonstrate that Tinagl1 constitutes an extraembryonic tissue-specific protein in the postimplantation period. Specifically, Tinagl1 forms a component of the Reichert's membrane that interacts with laminin-1 [17]. These results suggest that Tinagl1 most likely plays physically supporting role in embryo development.

In addition, we demonstrated that Tinagl1 is markedly expressed in the uterine decidua during the postimplantation period [19]. During the preimplantation period, Tinagl1 was expressed in the basement membranes of uterine luminal epithelial cells on days 1 and 2 of pregnancy (day 1 = vaginal plug), while its expression levels declined after day 3 [19]. The uterine expression levels of *Tinagl1* mRNA and Tinagl1 protein were similar on days 1 to 4 of pregnancy. In contrast, the expression of *Tinagl1* mRNA and Tinagl1 protein increased in postimplantation uteri [19]. From days 6 to 8, Tinagl1 was markedly expressed in the decidual endometrium. Tinagl1 bound to integrins α5 and β1 in the decidual endometrium [19]. These results suggest that uterine Tinagl1 functions during the postimplantation period; in particular, it associates with integrin α5β1 in the decidualized uterine endometrium [19]. Therefore, Tinagl1 is associated with the decidualization of the uterine endometrium during the postimplantation period.

Previous substantial progress in developmental biology and cancer biology have revealed similarities between early embryo development and tumorigenesis with respect to cell invasive behaviors, epigenetic regulation, gene expression, protein profiling and other important biological behaviors [20–22]. The tumor metastasis suppressor CD82 (also known as KAI1) is associated with both in uterine endometrial decidual cells and trophoblast cells during invasion of trophoblasts that leads to the early pregnancy stage [23–26]. Meanwhile, recent studies have revealed that Tinagl1 has a novel role associated with metastasis in cancer cells [27–29]. Therefore, Tingal1 may be associated with embryo development and/or the decidualization of the uterine endometrium during pregnancy.

Although our previous study suggested that Tinagl1 is likely involved in embryonic development and/or uterine functions during pregnancy, determining the role of Tinagl1 in embryonic development during early pregnancy by gene deletion was still required. Our initial expectation in the present study was that *Tinagl1*$^{-/-}$ embryos might be lethal. To address whether *Tinagl1*$^{-/-}$ pups could be obtained, we mated *Tinagl1*$^{flox/flox}$;*CAG*$^{-/-}$ females and *Tinagl1*$^{+/-}$;*CAG*$^{Cre/-}$ males. We also examined homologous matings of *Tinagl1*$^{-/-}$ females and *Tinagl1*$^{-/-}$ males and investigated reproductive phenotypes in Tinagl1-deficient females during pregnancy.

Materials and Methods

Generation of Tinagl1-deficient mice

A targeting vector was constructed using pNT1.1 containing the Neo-resistance gene (Neor) as a positive selection marker and a herpes simplex virus thymidine kinase gene (tk) as a negative selection marker. The *Tinagl1*-exon 3 targeting vector was constructed by flanking the exon with a *loxP* site and a *loxP* and FLP recombinase target (*FRT*) site-flanked Neor cassette (Fig. 1A). The targeting vector was electroporated into mouse EGR-G01 embryonic stem (ES) cells. Homologous recombinations were selected using G418. Chimeric mice derived from targeted ES cells were mated with C57BL/6NCr mice to obtain F1 *Tinagl1*$^{flox/+}$ mice (Fig. 1B). To generate a null *Tinagl1* allele, *Tinagl1*$^{flox/flox}$ mice were mated with *CAG-Cre* transgenic mice that ubiquitously express Cre recombinase (Fig. 1C). DNA obtained from tail biopsies was subjected to genotyping analyses by PCR with KOD FX DNA polymerase (Toyobo, Osaka, Japan) and the oligonucleotide primers 5′-CTTACCTGGGCTTCAGTTTCTTTCCTA-3′ (forward) and 5′-CTTCTAACCTACTTGGCTGGCACTCTAC-3′ (reverse) using the following PCR conditions: 5 min at 94 C for one cycle, followed by 20 sec at 94 C and 6 min at 68 C for 30 cycles. The primers were designed to generate PCR products of 3.2 kbp for the wild-type *Tinagl1* allele, 4.9 kbp for the floxed allele and 2.8 kbp for the knockout allele (Fig. 1B, C). Tinagl1 protein was detected by immunohistochemistry as described previously [16, 19] (Fig. 1D, E). Mice were bred in our animal care facility. All animal experimental procedures used in this study were performed in accordance with the instructions of the Guide for the Care and Use of Laboratory Animals published by Utsunomiya University.

Homologous mating

To address the reproductive ability of adult mice, homologous matings of *Tinagl1*$^{-/-}$ females: *Tinagl1*$^{-/-}$ males and *Tinagl1*$^{flox/flox}$ females: *Tinagl1*$^{flox/flox}$ males were examined. Two or three females (2–6 months old) were placed in a cage with a male (2–6 months old) in the late afternoon. These animals were housed together overnight. Females were checked every morning for the presence of a vaginal plug. If a plug was found, the male was removed from the cage, and the female was considered to be pregnant (day 1 = vaginal plug). These females were monitored for placental sign, birth and the numbers of dead and live pups. If a plug was not found, mating was continued until 3 weeks. Phenotypes of fertility were classified as types I to VI, as follows: type I, no vaginal plug (no coitus); type II, vaginal plug positive, but no placental sign; type III, vaginal plug and placental sign positive, but no delivery; type IV, dead at birth or fewer than 3 live pups; type V, live pups, but half of them died within 7 days; and type VI, normal.

Immunohistochemical analysis of Tinagl1

Immunohistochemical analysis was performed as previously described [19]. Frozen 10-µm sections from snap-frozen tissues were mounted onto silane-coated glass slides and stored at –80 C until used. Sections were fixed in cold acetone on ice, immersed in 3% hydrogen peroxide in methanol, and treated with 1% BSA in PBS. For the primary antibody reaction, sections were incubated with rabbit polyclonal antibody to Tinagl1. After washing, sections were

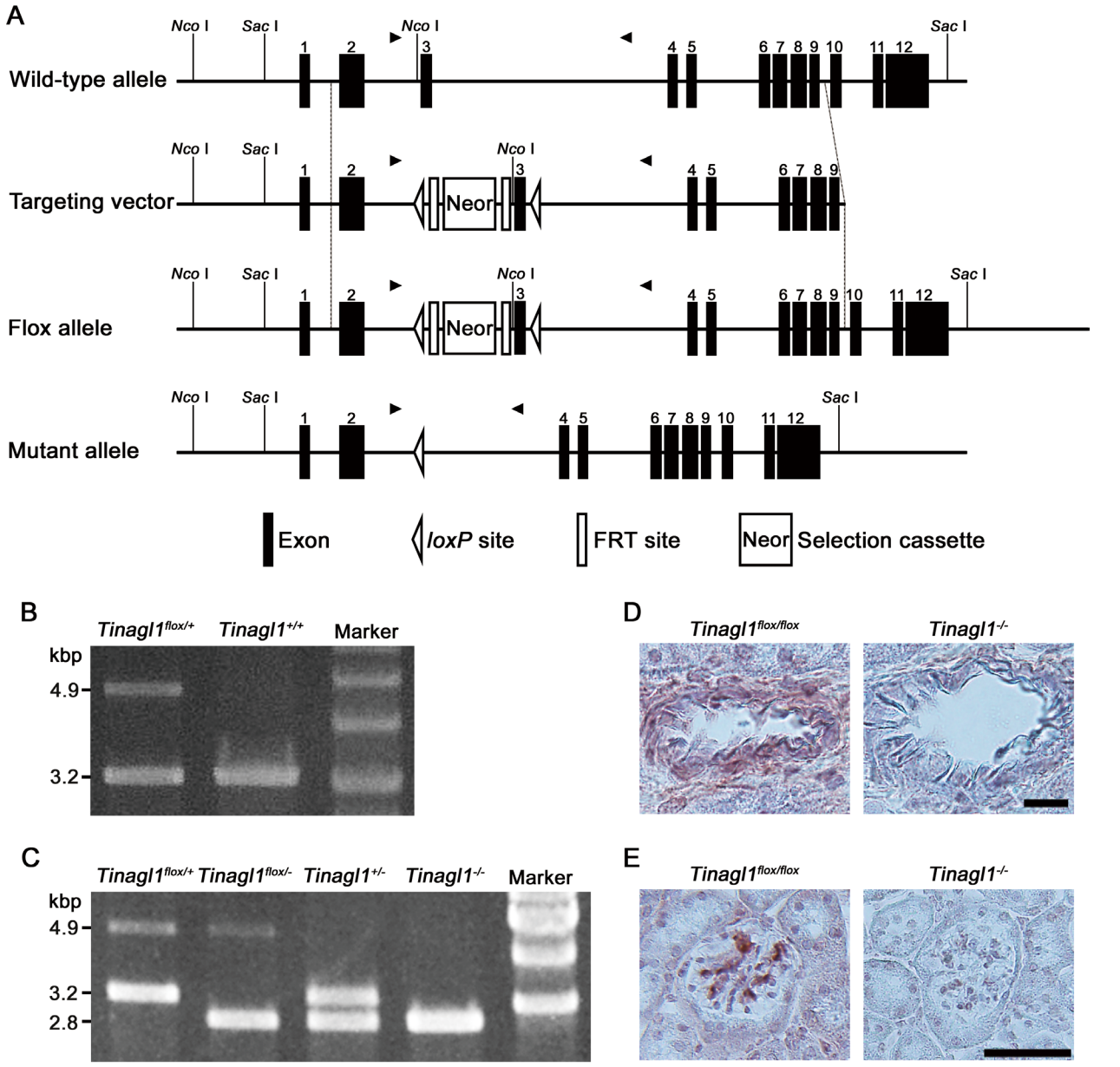

Fig. 1. Generation of *Tinagl1*-deficient mice. (A) Schematic representation of targeted disruption *via* homologous recombination of the targeting vector and recombination steps. The numbered closed boxes denote the translated exons of the gene. Arrowheads correspond to the positions of the primers used for detecting wild-type, Flox and mutant alleles. (B) PCR genotype analysis of pups generated by the mating of chimeric mice derived from targeted ES cells with C57BL/6NCr mice to obtain F1 *Tinagl1*$^{flox/+}$ mice. (C) PCR analysis of pups generated by matings between *Tinagl1*$^{flox/flox}$;*CAG*$^{-/-}$ females and *Tinagl1*$^{+/-}$;*CAG*$^{Cre/-}$ males. (D, E) Protein expression of Tinagl1 in the artery (D) and glomerulus in the kidney (E). Scale bars represent 30 μm.

incubated with biotinylated goat anti-rabbit antibody (Thermo Fisher Scientific, Waltham, MA, USA). After incubation with horseradish peroxidase-conjugated streptavidin (Thermo Fisher Scientific), reactions were visualized using 3-amino-9-ethylcarbazole (Thermo Fisher Scientific) as a chromogen, followed by counterstaining with hematoxylin. Reddish deposits indicated the sites of immunoreaction.

Superovulation, in vitro fertilization and embryo culture

Superovulation, *in vitro* fertilization and culture of embryos were performed as previously described [30–32]. Female mice were subjected to superovulation by intraperitoneal injection of 5 IU equine chorionic gonadotropin (eCG) (ASKA Animal Health, Tokyo, Japan) followed by 5 IU human chorionic gonadotropin (hCG) (ASKA Animal Health) 48 h later. Ovulated oocytes were then

collected in human tubal fluid (HTF) medium without phenol red (HTF-P) 14 h after hCG injection. Spermatozoa were obtained from *Tinagl1*$^{-/-}$ males and preincubated for 2–3 h in HTF-P to allow for capacitation; the final concentration was 700 spermatozoa/µl. Four hours after insemination, the oocytes were transferred into 100 µl of potassium simplex optimized medium (KSOM) without phenol red (KSOM-P), overlaid with paraffin liquid (Nacalai Tesque, Kyoto, Japan) and cultured in a humidified atmosphere with 5% CO_2 at 37 C.

Statistical analysis

A Chi-square test was used to evaluate differences in rates of types I to VI. Comparisons with expected values of less than 5 were analyzed using Fisher's exact probability test. The Student's *t*-test was used to evaluate differences in the number of pups per litter, the number of ovulated oocytes and the rate of embryo development. $P < 0.05$ was considered statistically significant.

Results

Targeted disruption of the Tinagl1 gene

To analyze the effect of Tinagl1 ablation *in vivo*, we used gene targeting to generate *Tinagl1*$^{-/-}$ mice (Fig. 1A). Chimeric male mice derived from targeted ES cells were mated with C57BL/6NCr female mice to obtain F1 *Tinagl1*$^{flox/+}$ mice (Fig. 1B). To generate *Tinagl1*$^{flox/flox}$ mice, F1 *Tinagl1*$^{flox/+}$ females were mated with F1 *Tinagl1*$^{flox/+}$ males. *Tinagl1*$^{flox/flox}$ mice were used to generate *Tinagl1*$^{-/-}$ mice as described in Materials and Methods (Fig 1C). Deletion of the Tinagl1 protein was confirmed by immunohistochemical analysis (Fig. 1D, E).

Tinagl1 is not required for full-term embryonic development

We initially expected that *Tinagl1*$^{-/-}$ embryos may be lethal. To address whether *Tinagl1*$^{-/-}$ pups could be obtained, a total of 11 matings between *Tinagl1*$^{flox/flox}$;*CAG*$^{-/-}$ females and *Tinagl1*$^{+/-}$;*CAG*$^{Cre/-}$ males were performed, generating 69 offspring with a normal average litter size of 6.3 ± 1.0 pups (mean ± SEM). The genotypes of the pups were determined (Fig 1C), and the frequencies are shown in Table 1. The relative birth frequency of homozygous mutant animals (*Tinagl1*$^{-/-}$) was 23.2% (16/69), which was roughly equal to the expected Mendelian distribution. These results indicate that *Tinagl1*$^{-/-}$ embryos are not lethal during development to term.

Impaired fertility in Tinagl1-null females during pregnancy

The breeding data described in the previous section revealed that *Tinagl1*-null embryos develop to term. However, it was unclear whether the reproductive ability in adult *Tinagl1*-null mice was normal. To address this issue, we examined homologous matings of *Tinagl1*$^{-/-}$ females: *Tinagl1*$^{-/-}$ males and *Tinagl1*$^{flox/flox}$ females: *Tinagl1*$^{flox/flox}$ males. Although *Tinagl1*$^{-/-}$ male mice were fertile, matings of *Tinagl1*$^{-/-}$ females with fertile *Tinagl1*$^{-/-}$ males for 7 generations resulted in various subfertility phenotypes (Table 2), which were classified as types I to VI. As shown in Table 2, 30.4% (42/138) of the matings exhibited infertility (types I to III); 8.0% (11/138) of the *Tinagl1*$^{-/-}$ females had no pups or < 3 live pups (type IV); 20.3% (28/138) of the females had live-born pups, but half of the pups died within 7 days (type V); and 41.3% (57/138) of the *Tinagl1*$^{-/-}$ females exhibited normal reproduction (type VI). The rate of type VI in the *Tinagl1*$^{-/-}$ females (41.3%) was lower than that in the *Tinagl1*$^{flox/flox}$ females (82.1%, $P < 0.05$), while the rate of types V in the *Tinagl1*$^{-/-}$ females (20.3%) was higher as compared with that in the *Tinagl1*$^{flox/flox}$ females (0%, $P < 0.05$). As shown in Table 3, the number of pups per litter in type VI *Tinagl1*$^{-/-}$ females (6.7 ± 0.2) did not differ from that in the type VI *Tinagl1*$^{flox/flox}$ females (6.5 ± 0.4; $P > 0.05$).

Tinagl1-deficient female mice exhibit normal ovarian responses to induced ovulation

Although adult *Tinagl1*-deficient female mice exhibit various subfertility phenotypes, the underlying cause of these defects is not clearly understood. We expected that ovarian function defects might be associated with subfertility; to examine this, superovulation was induced with eCG and hCG. As shown in Table 4, *Tinagl1*-null mice had 13.7 ± 1.3 ovulated oocytes and 12.6 ± 1.3 normal oocytes; the numbers were not significantly different from those of *Tinagl1*$^{flox/flox}$ mice ($P > 0.05$).

Fertilization and developmental competence of ovulated oocytes are normal in adult Tinagl1-deficient female mice

In vitro fertilization followed by embryo culture was employed to determine the competency of ovulated oocytes for fertilization and preimplantation development. Cleavage of fertilized eggs to the 2-cell stage or beyond was used as a parameter for successful *in vitro* fertilization and developmental competency. As shown in Table 5, 86.8 ± 2.1% of oocytes ovulated by *Tinagl1*$^{-/-}$ females showed successful fertilization. This rate was similar (92.0 ± 3.2%) to that observed for oocytes obtained from *Tinagl1*$^{flox/flox}$ mice ($P > 0.05$). Since the spermatozoa used for *in vitro* fertilization were obtained from *Tinagl1*$^{-/-}$ male, the genotypes of the fertilized eggs were *Tinagl1*$^{-/-}$ or *Tinagl1*$^{flox/-}$. The rates of *Tinagl1*$^{-/-}$ and *Tinagl1*$^{flox/-}$ embryos that developed into blastocysts were 73.2 ± 3.2% and 75.9 ± 5.9%, respectively. There were no significant differences between *Tinagl1*$^{-/-}$ and *Tinagl1*$^{flox/-}$ embryos at each 24-h stage ($P > 0.05$).

Discussion

Our previous studies suggested that Tinagl1 is associated with embryonic development and/or functions involved in the decidualization of the uterine endometrium during the postimplantation period in the mouse [17–19]. However, the present study revealed that *Tinagl1*$^{-/-}$ embryos were not lethal and could develop to term. *In vitro* fertilization followed by embryo culture also demonstrated normal developmental potential in *Tinagl1*-null embryos during the preimplantation period. Meanwhile, homologous matings of *Tinagl1*$^{-/-}$ females and *Tinagl1*$^{-/-}$ males resulted in impaired fertility phenotypes during pregnancy. These results suggest that Tinagl1 is related in part to fertility, fetal development and/or postnatal development.

The rate of normal fertility (type VI) in *Tinagl1*$^{-/-}$ females was lower than that in *Tinagl1*$^{flox/flox}$ females. In infertile category types I to V, the rate of type V in *Tinagl1*$^{-/-}$ females was higher as compared with that in *Tinagl1*$^{flox/flox}$ females. The type V phenotype did result in live pups; however, half of them died within 7 days. Although half of the pups died, the remaining half survived and grew. Therefore,

Table 1. Number and frequency (%) of pup genotypes from heterozygous matings between *Tinagl1*$^{flox/flox}$;*CAG*$^{-/-}$ females and *Tinagl1*$^{+/-}$;*CAG*$^{Cre/-}$ males

	Tinagl1$^{flox/+}$	*Tinagl1*$^{flox/-}$	*Tinagl1*$^{+/-}$	*Tinagl1*$^{-/-}$	Total
Females	15 (21.7)	8 (11.6)	8 (11.6)	10 (14.5)	41 (59.4)
Males	4 (5.8)	5 (7.2)	13 (18.8)	6 (8.7)	28 (40.6)
Total	19 (27.5)	13 (18.8)	21 (30.4)	16 (23.2)	69 (100)

The number of offspring from a total of 11 matings is reported for each group. Numbers in parentheses are the percentages of offspring in each group.

Table 2. Fertility in *Tinagl1* $^{flox/flox}$ or *Tinagl1*$^{-/-}$ females during pregnancy from homologous matings

Genotype		No. of mating examined	No. (%) of female mice					
Female	Male		Type I	Type II	Type III	Type IV	Type V	Type VI
Tinagl1$^{flox/flox}$	*Tinagl1*$^{flox/flox}$	28	3 (10.7)[a]	2 (7.1)[a]	0 (0)[a]	0 (0)[a]	0 (0)[a]	23 (82.1)[a]
Tinagl1$^{-/-}$	*Tinagl1*$^{-/-}$	138	24 (17.4)[a]	15 (10.9)[a]	3 (2.2)[a]	11 (8.0)[a]	28 (20.3)[b]	57 (41.3)[b]

Type I, no vaginal plug (no coitus); type II, vaginal plug positive, but no placental sign; type III, vaginal plug and placental sign positive, but no delivery; type IV, dead at birth or fewer than 3 live pups; type V, live pups, but half of them died within 7 days; type VI, normal. [a, b] Values with different superscripts within each column differ significantly ($P < 0.05$).

Table 3. Number of newborn pups from type VI *Tinagl1*$^{flox/flox}$ or type VI *Tinagl1*$^{-/-}$ matings

Genotype		No. of matings examined*	No. of pups per liter[†]
Female	Male		
Tinagl1$^{flox/flox}$ (Type VI)	*Tinagl1*$^{flox/flox}$	31	6.5 ± 0.4
Tinagl1$^{-/-}$ (Type VI)	*Tinagl1*$^{-/-}$	126	6.7 ± 0.2

* These include second or third matings. [†] Mean ± SEM.

Table 4. Ovulation in adult *Tinagl1*$^{-/-}$ female mice

Genotype	No. of mice examined	No. of ovulated oocytes*	No. of normal oocytes*
Tinagl1$^{flox/flox}$	18	11.4 ± 1.3	10.7 ± 1.3
Tinagl1$^{-/-}$	44	13.7 ± 1.3	12.6 ± 1.3

* Mean ± SEM.

Table 5. Developmental rates of *in vitro*-fertilized oocytes retrieved from *Tinagl1*$^{-/-}$ female mice

Genotype of retrieved oocytes	No. of mice examined	% of embryos developed to*			
		≥ 2-cell at 24 h	≥ 4-cell at 48 h	≥ morula at 72 h	≥ blastocyst at 96 h
Tinagl1$^{flox/flox}$	17	92.0 ± 3.2	88.6 ± 4.1	83.8 ± 4.9	75.9 ± 5.9
Tinagl1$^{-/-}$	43	86.8 ± 2.1	84.9 ± 2.2	80.4 ± 2.9	73.2 ± 3.2

* Mean ± SEM.

maternal behavior such sufficient feeding of the pups may be normal. Meanwhile, our results also indicate that *Tinagl1*$^{-/-}$ embryos can develop to term. Therefore, the type V phenotype in *Tinagl1*-null mice could be caused by aberrant perinatal development supported by insufficient maternal conditions during late pregnancy.

Ovulation induced by hormonal stimulation in *Tinagl1*-null female was not significantly different from that in *Tinagl1*$^{flox/flox}$ females. These results suggest that *Tinagl1*$^{-/-}$ female mice have normal ovarian functions, such as response to hormonal stimulation for follicular development and ovulation. Our results also indicate that *in vitro* fertilized *Tinagl1*-null embryos developed normally into blastocysts. These results suggest that the oocyte potential in *Tinagl1*$^{-/-}$ mice is normal and that Tinagl1 is not essential for either fertilization or preimplantation development *in vitro*.

Our results indicate that *Tinagl1* deficiency causes a variety of infertility phenotypes in adult females during pregnancy. Although the rates of types I to IV were not significantly different between *Tinagl1*$^{-/-}$ and *Tinagl1*$^{flox/flox}$ females, *Tinagl1* deficiency showed a tendency to increase the rates of types I to IV. Type I was the no vaginal plug (no coitus) phenotype. Type II was the phenotype with a vaginal plug but no placental sign. Placental sign is the appearance of blood in the vagina during the postimplantation period that can be attributed to leakage from the sinusoids formed in the mesometrial portion of the decidua into the uterine lumen [33, 34]. The results suggest that implantation was not successful in this phenotype, possibly due to the failure of embryonic preimplantation development and/or uterine dysfunction during the pre- and postimplantation periods. Type III was the phenotype with the placental sign on day 12 and/or 13 of pregnancy but without pup delivery. Therefore, since implantation and uterine decidualization during the postimplantation

period were successful, type III probably results from a loss of embryonic development during the postimplantation period caused by maternal dysfunction in the final trimester.

The type IV phenotype, dead at birth or fewer than 3 live pups, may be due to female dysfunction during late pregnancy or labor. Increased integrin expression and activation in the pregnant uterus are thought to be critical for the formation of the mechanical syncytium required for labor [35]. Tinagl1 is a ligand of several integrins, including α1β1, α2β1 and α5β1 [16]. Expression of integrins α1, α5 and β1 is upregulated in the rat myometrium during late pregnancy and labor [36, 37]. Furthermore, Tinagl1 is expressed in the uterine myometrium [19]. Therefore, Tinagl1 could be associated with integrin function, e.g. deficiency of Tinagl1 could cause dysfunction of integrins α1β1 and/or α5β1, resulting in the type IV phenotype.

Tinagl1 has been identified as a matricellular protein [16]. One feature of matricellular proteins is transient expression during specific developmental stages and pathological conditions rather than constitutive expression [38]. Indeed, several studies examining mice deficient in matricellular proteins have identified previously unsuspected consequences stemming from the lack of appropriate interactions between cells and their environment [14]. Therefore, the impaired fertility in *Tinagl1*-deficient female mice during pregnancy could be affected by an unstable physiological condition.

Interestingly, recent studies have revealed a novel role for Tinagl1 associated with metastasis in cancer cells [27–29]. Tinagl1 was confirmed to be a Sec23a-dependent metastasis suppressor by functional and clinical correlation studies [27]. The miR-200s, which function upstream of Tinagl1, are associated with increased risk of metastasis in breast cancer and promote metastatic colonization in mouse models, i.e. the miR-200s promote metastatic colonization partly through direct targeting of Sec23a, which mediates secretion of metastasis suppressive proteins, including Tinagl1 [27]. In mammary carcinomas, Tinagl1 is upregulated in highly metastatic and pro-metastatic tumors, which could indicate tumor-specific or context-dependent mechanisms of action [28]. Furthermore, bioinformatic analysis of potentially critical genes that may mediate non-small cell lung cancer (NSCLC) metastasis identified Tingal1 as a possible candidate for drug compounds that inhibit the expression of these genes [29]. Therefore, the altered expression of metastasis-associated proteins in *Tinagl1*-deficient mice could, under certain cellular, physiological and/or environmental conditions, lead to aberrant decidualization such as deciduoma formation or even the development of cancer, which may in turn be associated with subfertility in *Tinagl1*-null mice.

In conclusion, the present study describes new observations including subfertility phenotypes in *Tinagl1*-null female mice and the facts that *Tinagl1*$^{-/-}$ embryos could develop to term. Adult *Tinagl1*$^{-/-}$ females showed impaired fertility during pregnancy, including failure to carry pregnancy to term and perinatal lethality. However, the ovarian ovulation function induced by hormonal stimulation is distinct from the subfertility caused by *Tinagl1* deficiency. Furthermore, the oocyte potential in *Tinagl1*$^{-/-}$ mice is normal, and Tinagl1 is not essential for either fertilization or preimplantation development *in vitro*. Because the phenotypes of matricellular protein-deficient mice are often affected by the lack of appropriate interactions of the cells with their environment, investigation of disrupted cellular, physiological and/or environmental conditions may elucidate the role of Tinagl1 during pregnancy and its physiological role in female mice.

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