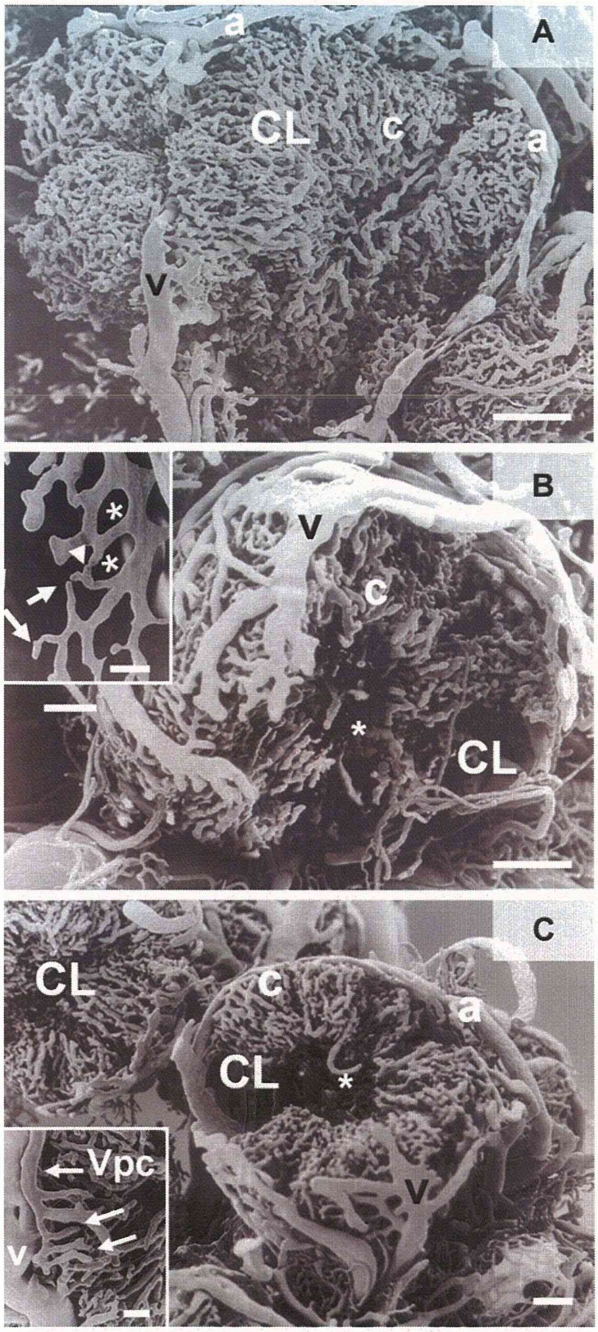

Fig. 4. Representative images of the outer surface of rat developing CLs by scanning electron microscopy of vascular corrosion casts. (A) Group 1: gonadotropin-treated Wistar-Imamichi rats (control). The dense vascular network of the CL is characterized by small caliber capillaries (c) growing toward the avascular central cavity. The developing CL is supplied by a system of arterioles (a) and drained by venules (v). Bar, 200 μm. (B) and (C) Group 3: thyroxine plus gonadotropin-treated *rdw* rats. Three luteal vascular plexuses in the ovarian cortex. An abundant superficial system of drainage, made of flattened venules (v), covers the great part of the plexuses. Note the presence of small arterioles (a) supplying the CLs. A copious network of capillaries (c) originates from these arterioles. Note also the presence of a poorly vascularized area in the center of CLs (asterisks). (B) Bar, 200 μm. (C) Bar, 100 μm. (B, inset) A portion of the luteal capillary network is seen at higher magnification: note the organization in large meshes (*). Numerous angiogenic figures, i.e., characteristic blood vessel formations indicating angiogenesis, such as sprouts (arrows) and vesicular buds (arrowheads) are present. Bar, 50 μm. (C, inset) A portion of the drainage system of a luteal vascular plexus in the ovarian cortex. The luteal capillaries are drained by small postcapillary venules (Vpc), which are collected by a wide system of large and flattened venules of drainage (v). From the Vpc often originated capillaries of neoformation in the form of blind endings of the resin (venular angiogenesis) (arrows). Note the tortuous course of the luteal capillary vessels. Arrows indicate sprouts. Bar, 50 μm.

revealed by the presence of a poorly vascularized area in the center of these plexuses (Fig. 4B, C). At higher magnification, tortuous and dilated capillaries (diameter 12–13 μm), arranged in irregular polygonal large meshes (ranging from 50 to 100 μm in size) formed a luteal capillary network (Fig. 4B, inset). These capillaries showed numerous sprouting angiogenic figures such as buds and sprouts of the resin (Fig. 4B, inset). CL capillaries were drained by small postcapillary venules (diameter 20–40 μm) (Fig. 4C, inset). These venules often originated capillaries of neoformation in the form of blind endings of the resin (venular angiogenesis). Postcapillary venules were collected by a wide superficial system of large and flattened venules of drainage (diameter 80–120 μm) (Fig. 4C, inset).

3.2. Quantitative study of pericytes

The number (mean ± SD) of follicular and luteal capillaries did not vary among the three experimental groups ($P > 0.05$; Table 1). The number of pericytes (mean ± SD) in CL capillaries of T4 plus gonadotropin-treated *rdw* rats (group 3) was similar to that found in control CLs. CL capillaries were not present in group 2 (Table 1). Pericytes in follicular capillaries significantly decreased in group 2, compared with control rats and group 3 ($P < 0.001$). The total number of pericytes was significantly higher in groups 1 and 3 than in group 2 ($P < 0.001$; Table 1).

3.3. Molecular study

Thyroxine treatment significantly increased mRNA expression levels of VEGF and bFGF in group 3 ovaries (containing many CLs), when compared with those of group 2 (where no CL was found in the ovaries), and showed values comparable with those obtained in group 1 (Fig. 5). More specifically, VEGF mRNA expression, when compared with group 1 (relative ratio ± SD, 10.167 ± 0.764), significantly decreased in group 2 (8.333 ± 0.289; $P < 0.05$) and increased in group 3 (9.5 ± 0.5; $P < 0.05$). Similarly, bFGF mRNA expression significantly decreased in group 2 (5.333 ± 0.557) compared with group 1 (7.633 ± 0.551; $P < 0.01$). Thyroxine (group 3) restored an expression pattern of bFGF similar to those found in group 1 (7.967 ± 1.05; $P > 0.05$; group 2 vs. group 3: $P < 0.05$). No differences were detected in the other growth factors analyzed (TGFβ-1, EGF, TNFα) ($P > 0.05$) (Fig. 5A, B).

4. Discussion

Thyroxine supports fertility restoration in immature infertile spontaneous hypothyroid *rdw* rats by inducing, in combination with gonadotropins, functional changes

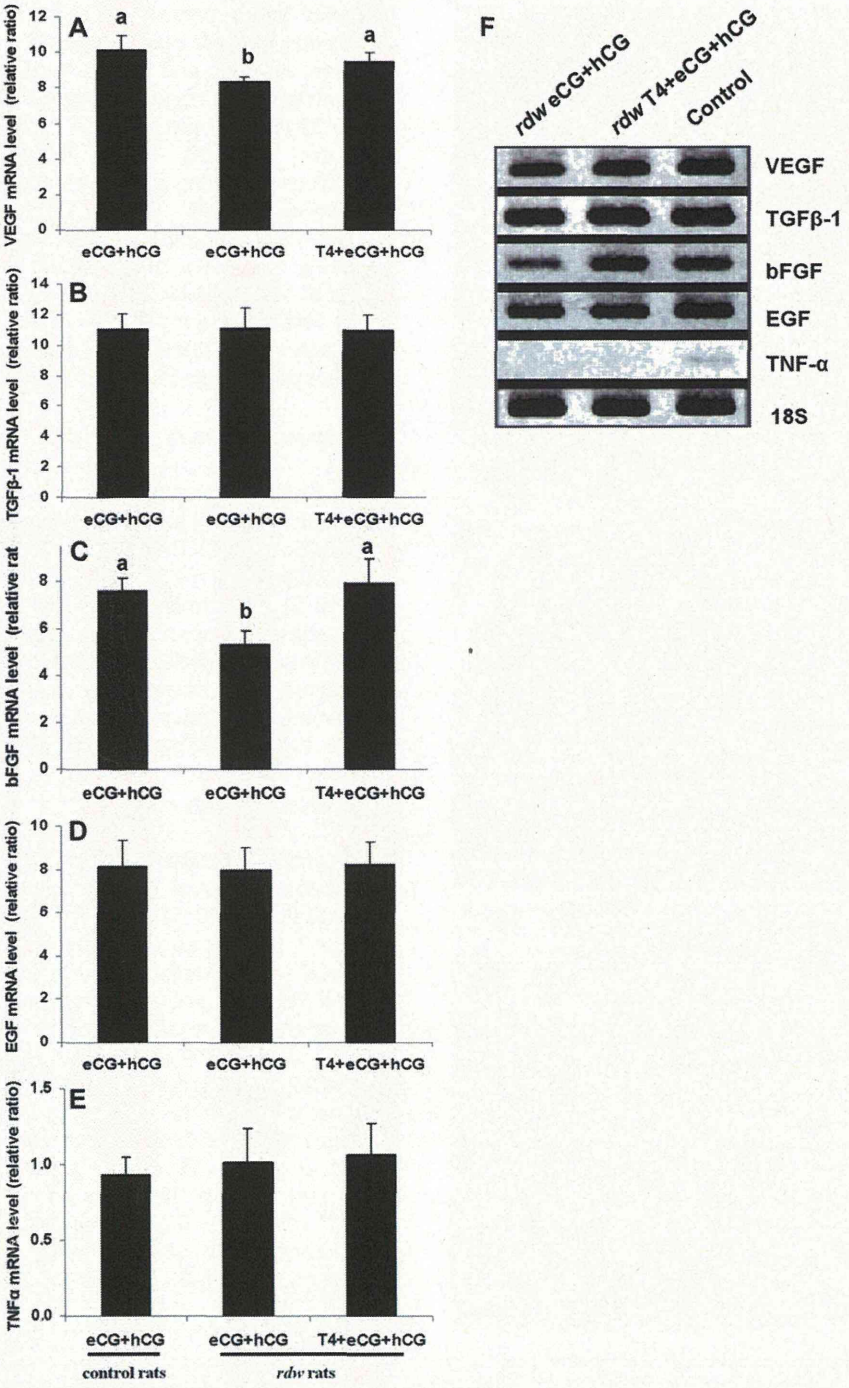

Fig. 5. mRNA expression pattern of growth factors involved in the regulation of angiogenesis. (A) Expression of mRNA for vascular endothelial growth factor (VEGF) (A), transforming growth factor (TGF)β-1 (B), basic fibroblast growth factor (bFGF) (C), epidermal growth factor (EGF) (D), tumor necrosis factor (TNF)-α (E) by reverse transcriptase polymerase chain reaction (RT-PCR) in ovaries of: group 1, control rats; group 2, gonadotropin-treated *rdw* rats; group 3, thyroxine plus gonadotropin-treated *rdw* rats. Results are representative of three separate experiments on whole ovaries obtained from two animals per each experimental group. Data are represented as mean ± SD of the relative ratio between each angiogenic factors and 18S RNA. Significant differences are represented by small letters ($P < 0.05$). (F) A representative image of RT-PCR is shown.

such as increased follicular development, augmented plasma estradiol levels [25], and improved percentage of fertilized eggs producing live pups [24,38]. It also promotes angiogenesis in ovarian FLs of *rdw* rats by upregulating mRNA expression of major angiogenic factors [19].

Because angiogenesis has a key role in the correct accomplishment of the ovarian function [1–5,8–10,19,

32,39,40], we aimed to study the effects of T4 and gonadotropin administration on postovulatory microcirculation and angiogenesis in female *rdw* rats. The results obtained showed for the first time that T4, in combination with eCG and hCG, modulated CL angiogenesis by the upregulation of angiogenic growth factors expression, such as VEGF and bFGF, associated with endothelial cell and pericyte activation and proliferation.

Gonadotropins are the primary factors with angiogenic potentiality—involved in FL development and CL formation [17]—interacting with thyroid hormones. Experimental studies performed *in vivo* and *in vitro* demonstrated that T4 is able to act as a "biological amplifier" of the action exerted by FSH in the functional differentiation of granulosa cells at several levels. In particular, T4 cooperates with hypophysary gonadotropins in promoting granulosa cell morphological differentiation, LH receptor acquisition, and steroidogenic enzyme (3β-hydroxysteroid dehydrogenase and aromatase) synthesis [21,41–43]. However, the fact that not only granulosa cells but also stromal cells, cumulus cells, and oocytes of different mammals express thyroid hormone receptors [41–44], suggests a wider action field of thyroid hormones in the ovary, involving factors such as integrins and growth factor receptors. Thyroxine preferentially binds αVβ3 integrins to stimulate mitogen-activated protein kinase (MAPK)-dependent angiogenesis [45,46]. Integrin αvβ3, the vitronectin receptor, is expressed by vascular cells and is upregulated during sprouting angiogenesis [47]. It has been shown that αVβ3 integrin interacts with growth factor receptors such as VEGF receptor 2, fibroblast growth factor (FGF) receptor 3, epidermal growth factor receptor and platelet-derived growth factor receptor [48], all triggering the angiogenesis signaling cascade. Thyroxine, indeed, exerts the proangiogenic action that we observed in this study by activating αVβ3 integrins, which cross-talk with adjacent VEGF and bFGF receptors [29]. Moreover, T4 could probably also exert an action on pericyte recruitment and activation by interacting with the platelet derived growth factor receptor of pericytes, whose mRNA expression increased after hCG-induced ovulation [49].

In this study, the evidence of a combined angiogenic action of T4 and gonadotropins was proved by the growth of developing CLs provided with an extensive capillary network only in the T4 plus gonadotropin group, as occurred in control rats. Morphologic data showed several activated capillaries with angiogenic figures (budding and sprouting) in the CL parenchyma, and they were associated with numerous pericytes enveloped in a basal membrane that is continuous with the endothelial basal membrane [50]. The endothelium of growing luteal capillaries evidenced typical angiogenic ultrastructural parameters, such as highly variable shape, increment of plasma membrane specializations, and presence of irregularly shaped nuclei. Moreover, budding and sprouting of capillaries from preexisting vessels are considered to be proliferative (angiogenic) features [4,5,33,34]. The microvascular activation after T4 supplement was so intense that it involved the entire microvascular compartment of the CL, as shown by the presence of numerous arterioles and venules with voluminous and swollen smooth muscle cells. The growth of these bigger vessels serves to supply an adequate blood flow necessary to sustain the increased metabolic needs of the growing CL [9,10].

In presence of T4 (group 3) the total number of follicular and luteal pericytes (determined considering that the CL capillaries are formed from the invasion of thecal capillaries into the avascular antral cavity of the FL as a consequence of the angiogenic process [36]) around the activated endothelial cells of *rdw* rats were significantly higher than in gonadotropin-treated *rdw* rats (group 2) and comparable with those found in control rats, as confirmed by the quantitative analysis. These results further evidenced that T4 exerts a proangiogenic action by activating the recruitment of pericytes, essential in the first hours after ovulation [14,15,51,52]. Pericytes act by modulating the migration of thecal endothelial cells in the postovulatory FL wall, to organize the neoformation and maturation of luteal capillaries [3,14,53]. The activity of pericytes is particularly intense in developing CLs where they secrete intercellular vesicles, then convert into empty "spikes" releasing their content (growth factor and/or cytokine) after reaching target cells [16].

Numerous experimental results demonstrated that crucial events such as folliculogenesis, follicular atresia, luteogenesis, and luteolysis are locally regulated by angiogenic factors produced by different ovarian cell populations (thecal cells, granulosa cells, luteal cells). Ovarian angiogenic factors mainly belong to VEGF and FGF families of proteins [13,52,54–59]. During the early luteal stages [54], VEGF is actively expressed by luteal granulosa cells in both nonprimate and primate CL [13,53–55]. Vascular endothelial growth factor, which is induced both by the hypoxic environment in the ovulated FL and by hCG [56], plays a well established role as stimulator of endothelial cell proliferation and activator of antiapoptotic pathways [57]. Another key regulator of luteal angiogenesis is FGF [58] and, analogously to VEGF, its production in luteinizing granulosa cells increases in the early stages of luteal development [52], specifically during endothelial cell sprouting and tubule initiation [59].

The molecular data obtained by RT-PCR demonstrated that T4 plus gonadotropins allow the expression of luteal angiogenic factors in *rdw* rats and restore an expression pattern overlapping that of gonadotropin-primed animals (control rats). In particular, the T4 plus gonadotropin administration significantly increased mRNA expression levels of the two main growth factors regulating luteal angiogenesis, VEGF and bFGF. The lack of development of CLs in *rdw* rats treated only with gonadotropins is a direct consequence of the reduced expression of VEGF and bFGF, as shown in cow [60], where the CL growth was significantly impaired after neutralization of VEGF and bFGF. Remarkably, the increased expression of VEGF and bFGF mRNAs observed in T4 plus gonadotropin-treated *rdw* rats is in agreement with data obtained in cow developing CL [15], where the endothelial cells formed tubules under the influence of VEGFA and FGF2.

Compared with data previously obtained in ovarian FLs treated with T4 [19], relative ratios for the examined angiogenic factors increased in developing CL except for TNFα. These results are in agreement with data present in

literature, showing that though VEGF, TGFβ-1, bFGF, and EGF positively correlated with hCG [17,61,62], TNFα expression is attenuated by hCG [63].

Our morphologic data, especially those from SEM of vcc, clearly confirmed that mRNAs of the studied angiogenic factors are hereafter translated to become functionally active, as demonstrated by the formation of the rich and angiogenically active microvascular network only in the T4-treated group. Similar to what was found in control rats in the ultrastructural study, activated capillaries, arterioles and venules—often accompanied by the presence of recruited pericytes and swollen smooth muscle cells, respectively—were frequently observed in the developing CL of group 3 rats. On the contrary, in gonadotropin-treated *rdw* rats (group 2) any developing CL was found and several atretic FLs were present in the ovaries. Quiescent endothelial cells, rarely surrounded by pericytes provided with short cytoplasmic extensions, were observed. From these results it is indeed clear that the presence of an adequate hormonal supply, as already demonstrated in other experimental models [33,64], supports the physiologic evolution of FL and CL development associated with an intensive angiogenesis that, with dynamic remodeling, sustains the fast evolution of the quiescent early periovulatory FL in a highly vascularized CL [65].

5. Conclusions

Morphologic, quantitative, and molecular data clearly indicated for the first time that T4 plays an important role in stimulating luteal angiogenesis in gonadotropin-primed hypothyroid *rdw* rats. The early CL development is associated with a T4-mediated induction of luteal angiogenesis involving pericyte activation and upregulation of major growth factors implicated in the regulation of angiogenesis, such as VEGF and bFGF. The molecular mechanisms involved in these morphologic differentiations need to be further investigated even if these data confirmed that the consolidation of a well developed vascular network is necessary for the early CL development [19,36].

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Distribution of tubulointerstitial nephritis antigen-like 1 and structural matrix proteins in mouse embryos during preimplantation development *in vivo* and *in vitro*

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Summary

Tubulointerstitial nephritis antigen-like 1 (TINAGL1) is a novel matricellular protein that interacts with structural matrix proteins and promotes cell adhesion and spreading. We have previously reported unique localization of TINAGL1 to the trophectoderm (TE) of mouse blastocysts. TINAGL1 was found to be upregulated in implantation-competent blastocysts after estrogen treatment using progesterone-treated delayed-implantation models. Moreover, colocalization of TINAGL1 and extracellular matrix (ECM) protein laminin 1 was detected in the Reichert membrane on embryonic days 6.5 and 7.5. Although these data suggested a role for TINAGL1 in the embryo development at postimplantation, its relevance to other ECM proteins during preimplantation development is not clear. In this study, we examined the expression of TINAGL1 and its relevance to other ECM proteins fibronectin (FN) and collagen type IV (ColIV) during *in vivo* development of preimplantation embryos, particularly at blastocyst stage in detail. Localizations of TINAGL1, FN, and ColIV were similar. In 1-cell to 8-cell embryos, they were expressed in cytoplasm of blastomeres, and in morulae they were localized in the outer cells. FN and ColIV were expressed primarily on outer surface of the cells. In blastocysts, FN and ColIV were distributed in the cytoplasm of TE, but, just prior to implantation, they became localized uniquely to the blastocoelic surface of TE. In *in vitro* fertilized (IVF) blastocysts, expression levels of TINAGL1 and FN were lower than in *in vivo* blastocysts. These results suggest that, during preimplantation development, TINAGL1 may be involved in roles of structural matrix proteins, whose expression in blastocysts may be affected by *in vitro* culture.

Keywords: Collagen Type IV, Fibronectin, Preimplantation development, TINAGL1, Trophectoderm

Introduction

The fertilized oocyte first undergoes a series of early cleavage divisions to produce increasing numbers of progressively smaller cells, known as blastomeres, without changing the overall size of the embryo; it spans compaction and morula formation, and finally, cavitation with formation of a blastocyst. During mouse preimplantation embryo development, the 1-cell embryo develops into a blastocyst, a process that takes 4 days. In the mouse, the first sign of the attachment reaction (apposition stage) in the process of implantation occurs in the evening on day 4 of pregnancy (day 1 = vaginal plug) (Dey *et al.*, 2004; Wang & Dey, 2006). Estrogen secretion around noon on day 4 of pregnancy is essential for on-time blastocyst activation for implantation (Yoshinaga & Adams, 1966; Paria *et al.*, 1993). However, the mechanisms that regulate preimplantation embryo development are not fully understood.

Tubulointerstitial nephritis antigen-like 1 (TINAGL1; also known as adrenocortical zonation factor

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1 [AZ-1] or lipocalin 7) is a secretory protein of 52 kD polypeptides that was cloned from mouse adrenocortical cells and is known to be closely associated with the zonal differentiation of adrenocortical cells (Mukai *et al.*, 2003). Recently, we demonstrated the expression and localization of TINAGL1 in periimplantation mouse embryos (Igarashi *et al.*, 2009). Just prior to implantation at 23:00 h on day 4 of pregnancy, TINAGL1 was uniquely distributed in the blastocysts. Specifically, TINAGL1 was localized to the blastocoel site surface of the trophectoderm (TE) in implantation-competent (activated) blastocysts. This blastocyst activation can be initiated rapidly by a single injection of estradiol-17β (E_2) into ovariectomized and progesterone-primed pregnant mice, and is known as the delayed-implantation model (Yoshinaga & Adams, 1966; Psychoyos, 1973). In fact, we have also demonstrated the same localization and increased expression of TINAGL1 in activated blastocysts after E_2 treatment using this mouse model (Igarashi *et al.*, 2009). This unique localization may indicate a physiological role for TINAGL1 in the preparation of the blastocyst for successful implantation and/or subsequent pregnancy. We also demonstrated that, at postimplantation, TINAGL1 is a novel component of the Reichert membrane and interacts with laminin (LN) 1, and most likely plays a physical and physiological role in embryo development (Igarashi *et al.*, 2009). However, the relationship between TINAGL1 and other structural extracellular matrix (ECM) molecules in the mouse embryo, including fibronectin (FN) and collagen type IV (ColIV), is not clear.

ECM is present in every tissue but is most highly enriched in connective tissue and basement membrane (BM). ECM provides physical support to tissues and organs by occupying the space between cells. FN is a major constituent of ECM that promotes cell adhesion, spreading migration, and cytoskeletal organization (Hynes, 1990). ColIV is largely considered to be a structural component of BM, where it forms a scaffold with which other BM components, such as LN or FN, can associate (Laurie *et al.*, 1986), and it also mediates various cell functions directly (Murray *et al.*, 1979; Rubin *et al.*, 1981; Aumailley & Timpl, 1986). Some *in vitro* experiments suggest that FN promotes trophoblast adhesion, which may restrict migration, while other studies indicate that it facilitates motility (Burrows *et al.*, 1993; Damsky *et al.*, 1994; Irving *et al.*, 1995; Stephens *et al.*, 1995; Yelian *et al.*, 1995). ColIV can also support the outgrowth of primary trophoblast from the mouse blastocyst (Armant *et al.*, 1986; Sutherland *et al.*, 1988). Although some investigations into the expression of FN (Zetter & Martin, 1978; Wartiovaara *et al.*, 1979; Yohkaichiya *et al.*, 1988) and ColIV (Leivo *et al.*, 1980; Sherman *et al.*, 1980) in preimplantation embryos have been performed, detailed analyses of their expression from blastocyst activation to just prior to implantation have not been conducted.

In this study, we compared the immunocytological distributions of TINAGL1, FN, and ColIV during mouse preimplantation development, particularly in blastocysts at three stages: (1) before estrogen secretion; (2) after estrogen secretion; and (3) just prior to implantation. Importantly, it is known that *in vitro* culture (IVC) of preimplantation embryos alters their global gene expression patterns (Rinaudo & Schultz, 2004; Rinaudo *et al.*, 2006) and affects the behavior of mice after birth (Ecker *et al.*, 2004; Fernandez-Gonzalez *et al.*, 2004). Therefore, we compared further their expression in *in vivo* and *in vitro* fertilized (IVF) blastocysts.

Materials and methods

Animals

All ICR mice were purchased from Japan SLC Inc. (Shizuoka, Japan), and housed under controlled temperatures (22–27°C) with a constant photoperiod (13L–11D). Mice were provided with a pelleted diet (Oriental Yeast Co. Ltd., Japan) and water *ad libitum*. All investigations were performed in accordance with the Guide for Care and Use of Laboratory Animals of the Graduate School of Agricultural Science, Tohoku University.

In vivo embryo collection

Preimplantation embryos were collected as described previously (Igarashi *et al.*, 2009). In brief, female mature mice were mated with fertile males to induce pregnancy (day 1 [10:00 h] = vaginal plug). Removed oviducts or uteri were flushed with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS, Nissui Pharmaceutical Co., Ltd., Japan) containing 0.1% polyvinyl alcohol (PVA, Sigma). Preimplantation embryos were collected at the following stages during pregnancy: 1-cell, 2-cell, 4-cell, 8-cell, morula, and blastocyst on days 1 (21:00 h), 2 (10:00 h), 3 (01:00 h), 3 (10:00 h), 3 (21:00 h), and 4 (10:00 [before estrogen secretion], 18:00 [after estrogen secretion], and 23:00 h [just prior to implantation]), respectively.

In vitro fertilization and embryo culture

In vitro fertilization and embryo culture were performed as described previously (Matsumoto *et al.*, 2001; Hoshino & Sato, 152 2008). Immature mice were superovulated by subcutaneous injection with 5 IU of pregnant mare serum gonadotropin (PMSG;

ASKA Pharmaceutical Co., Ltd, Japan) at 3 weeks of age, followed by injection of 5 IU of human chorionic gonadotropin (hCG; Yell Pharmaceutical Co., Ltd, Japan) 48 h later. Mice were killed by cervical dislocation and oviducts were removed at 14 h post-hCG injection. Mature male mice over 8 weeks of age were killed by cervical dislocation and the epididymis was removed and carefully blotted free of blood and adipose tissues. Cauda epididymis was cut with fine scissors and the sperm droplet was scooped out with a 26-gauge needle (Terumo Co., Japan) and immediately transferred to a 200 μl drop of human tubal fluid (HTF) medium covered with mineral oil (Nacalai Tesque, Japan). Capacitation was allowed to proceed for 2–3 h at 37 °C in 5% CO_2 in humidified air. Collected cumulus cell–oocyte complexes (COCs) were moved to the HTF medium; the final concentration was 700 spermatozoa/μl. At 4 h after insemination, oocytes were cultured in a 100 μl drop of potassium simplex optimized medium (KSOM) overlaid with mineral oil in a humidified atmosphere of 5% CO_2 in air at 37°C. At 120 and 144 h after embryo culture, blastocysts were collected for immunostaining or western blotting.

Blastocyst stage developed *in vivo* and *in vitro*

Animal studies using the mouse model have demonstrated that after blastocoel formation in the morning on day 4 of pregnancy, the blastocysts are activated *in utero* around noon of day 4 for successful implantation to occur (Paria *et al.*, 1993), when an estrogen secretion takes place (Nilsson, 1966). Furthermore, just prior to implantation, activated blastocysts have a morphologically distinct structure (reviewed in ref. (McRae & Church, 1990)). In this study, the typical stages of *in vivo* blastocysts were as follows: pre-expansion (at 10:00 h before estrogen secretion); from expanded to hatched (at 18:00 h after estrogen secretion, referred to below as peri-hatching); and implantation-competent (at 23:00 h just prior to implantation, defined below as activated). In contrast, we collected IVF blastocysts following embryo culture for 120 or 144 h, because their most typical stages at each time point were pre-expansion or peri-hatching.

Immunostaining of embryos

Immunostaining of preimplantation embryos was performed as described previously (Matsumoto *et al.*, 2004; Li *et al.*, 2007; Igarashi *et al.*, 2009), with slight modifications. In brief, preimplantation embryos were fixed in 3.7% formaldehyde (Wako Pure Chemical Industries, Ltd., Japan) in PBS containing 0.1% PVA at room temperature for 30 min, and permeabilized with 0.25% Triton X-100 (Wako) in PBS containing 0.1% PVA for 5 min. After washing three times with PBS containing 0.1% PVA, embryos were incubated with rabbit anti-TINAGL1 polyclonal antibody (diluted 1:200), which was prepared as described previously (Li *et al.*, 2007), rabbit anti-fibronectin polyclonal antibody (diluted 1:50; Sigma), or rabbit anti-collagen type IV polyclonal antibody (diluted 1:50; Chemicon) overnight at 4°C. Following washes three times with PBS containing 0.25% Triton X-100 and 0.1% PVA, embryos were incubated with Alexa Fluor 488 goat anti-rabbit IgG (dilution 1:200, Invitrogen) for 1 h at room temperature. Washed three times with PBS containing 0.25% Triton X-100 and 0.1% PVA, nuclei were labelled with 10 μg/ml propidium iodide (Sigma) for 1 h at room temperature. After three washes, embryos were viewed using a Bio-Rad MRC-1024 confocal scanning laser microscope mounted on an Axioplan Zeiss microscope.

Western blotting

Western blot analysis was performed as described previously (Igarashi *et al.*, 2009). In brief, collected blastocysts were solubilized in 2× SDS sample buffer (0.5M Tris–HCl [Sigma] at pH 6.8, 10% β-mercaptoethanol [Wako], and 20% glycerol [Wako]). Electrophoresis was performed with 50 blastocysts in each lane on 12% polyacrylamide gels, and the resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporate Headquarters, MA). Thereafter, the membranes were blocked for 1 h at room temperature with Tris-buffered saline (TBS) containing 0.1% Tween 20 (Wako) (TBS-T) and 5% skimmed milk (Wako). Membranes were next incubated with rabbit anti-TINAGL1 polyclonal antibody (diluted 1:2000) overnight at 4 °C. Then, the membranes were reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:40,000) for 1 h at room temperature. Peroxidase activity was visualized using the ECL Plus western blotting detection system (GE Healthcare, Ltd., UK).

Results

Distribution of TINAGL1, FN, and ColIV in 1-cell embryos to morulae

To compare the cellular localization of TINAGL1 with that of FN and ColIV in preimplantation embryos, immunostaining was performed. In 1-cell to 8-cell embryos before compaction, TINAGL1 was localized in the cytoplasm. However, in compacted morulae, TINAGL1 was primarily localized in the outer cells. FN and ColIV were also expressed in the cytoplasm, but were more strongly distributed on the outer surface of the blastomeres than in the cytoplasm in

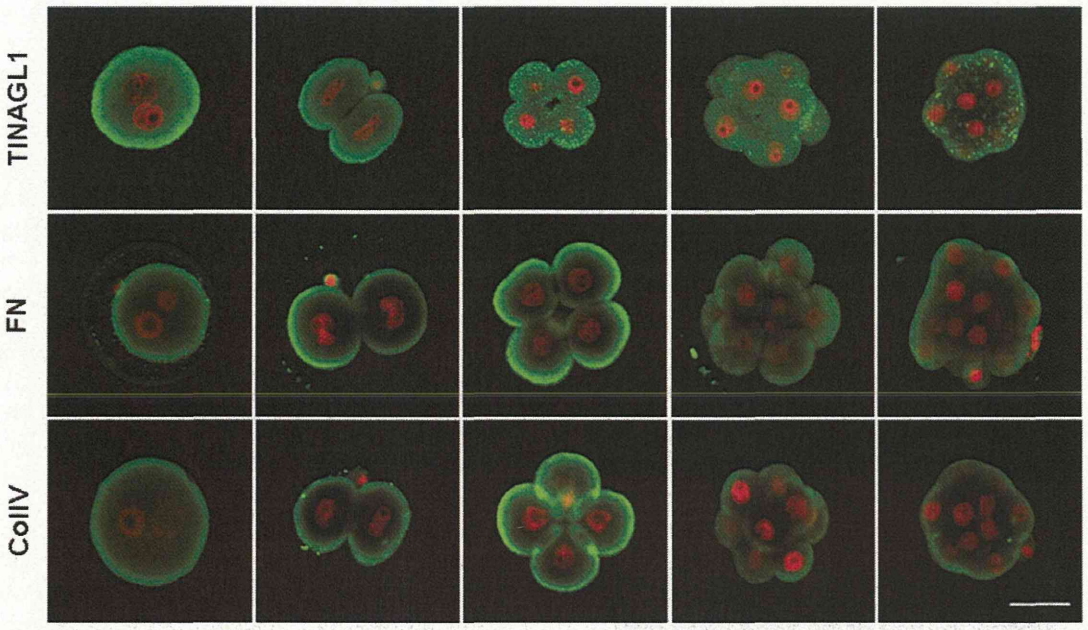

Figure 1 Distribution of TINAGL1, FN, and ColIV in 1-cell embryo to morula. Collected *in vivo* embryos (1-, 2-, 4- and 8-cell embryos, and compacted morulae) were immunostained for each protein with Alexa Fluor 488 (green). Red shows nuclei. Upper panel: TINAGL1. Middle panel: FN. Lower panel: ColIV. Bar = 50 μm. (See online for a colour version of this figure.)

1-cell to 8-cell embryos. In morulae, TINAGL1, FN, and ColIV were expressed in the outer cells (Fig. 1).

Distribution of TINAGL1, FN, and ColIV in blastocysts

In blastocysts, TINAGL1 was expressed in the cytoplasm of TE cells before (10:00 h) and after (18:00 h) estrogen secretion. Importantly, just prior to implantation (23:00 h), the distribution of TINAGL1 changed from the cytoplasm to the inner (blastocoelic) surface of TE cells. Similar to TINAGL1, FN and ColIV were also localized in the cytoplasm of TE in blastocysts before and after estrogen secretion. Just prior to implantation, their distribution patterns changed and they were localized mainly at the inner surface of TE. ColIV was also expressed in the cytoplasm of the inner cell mass (ICM) (Fig. 2).

Expression level of TINAGL1 in blastocyst developed *in vivo* and *in vitro*

As shown in Fig. 3, the expression of TINAGL1 in IVF blastocysts after 120 h of embryo culture was lower than that in *in vivo* blastocysts at 10:00 h, indicating that the expression of TINAGL1 in blastocysts at pre-expansion stage was lower in IVF than in *in vivo* blastocysts. Thereafter, the expression increased in both *in vivo* and IVF blastocysts (at 23:00 h in *in vivo* blastocysts and at 144 h in IVF blastocysts).

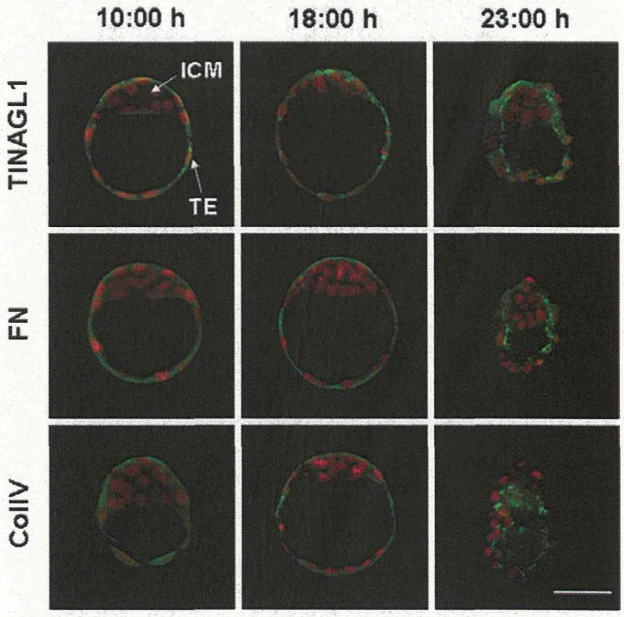

Figure 2 Distribution of TINAGL1, FN, and ColIV in mouse blastocyst. *In vivo* blastocysts were collected at 10:00 h (before estrogen secretion), 18:00 h (after estrogen secretion), and 23:00 h (just prior to implantation) on day 4 of pregnancy and immunostained for each protein with Alexa Fluor 488 (green). Red shows nuclei. Upper panel: TINAGL1. Middle panel: FN. Lower panel: ColIV. ICM: inner cell mass. TE: trophectoderm. Bar = 50 μm. (See online for a colour version of this figure.)

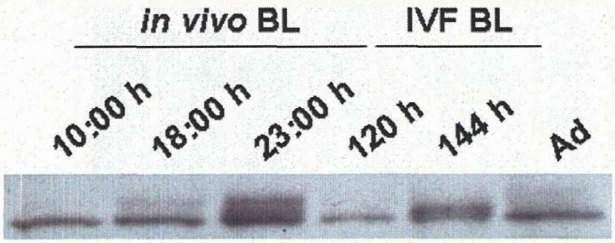

Figure 3 Expression of TINAGL1 in mouse blastocyst. *In vivo* blastocysts (BL) were collected at 10:00 h, 18:00 h, and 23:00 h on day 4 of pregnancy. IVF blastocysts were collected at 120 h and 144 h after embryo culture. Ad: adrenal gland as positive control expressing TINAGL1.

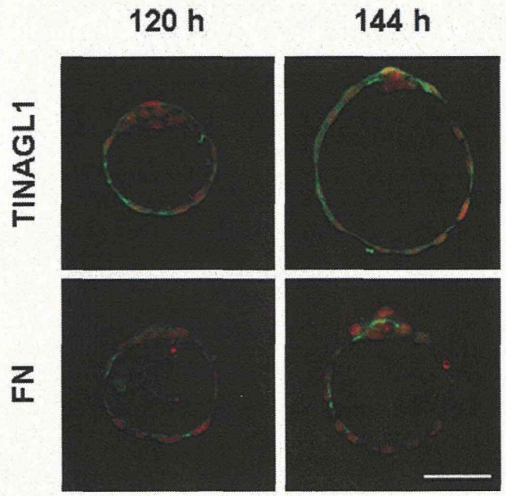

Figure 4 Distribution of TINAGL1 and FN in mouse IVF blastocyst. IVF blastocysts collected at 120 or 144 h after embryo culture were immunostained for each protein with Alexa Fluor 488 (green). Red shows nuclei. Upper panel: TINAGL1. Lower panel: FN. Bar = 50 μm. (See online for a colour version of this figure.)

Distribution of TINAGL1 and FN in IVF blastocysts

TINAGL1 and FN were localized in the cytoplasm of TE cells in IVF blastocysts (Fig. 4). Immunostaining showed that the expression of FN in IVF blastocysts was lower than that in *in vivo* blastocysts (refer Fig. 2, middle panel).

Discussion

In blastocysts, the localizations of FN and ColIV were similar to that of TINAGL1 in the TE, except for the strong expression of ColIV in the ICM. Importantly, these three proteins were distributed at the blastocoelic surface of the TE just prior to implantation. It is known that the TE secretes a BM; several molecules have been localized to the TE-generated BM, including FN, LN, ColIV, and heparin sulfate proteoglycans (Wartiovaara *et al.*, 1979; Leivo *et al.*, 1980; Carnegie, 1991; Thorsteinsdottir, 1992; Hierck *et al.*, 1993; Salamat *et al.*, 1993). *In vitro* outgrowth assays have demonstrated that the cellular attachment and outward migration were activated on FN (Bartlett & Menino, 1995; Schilperoort-Haun & Menino, 2002a,b) and, to a greater extent, on ColIV (Carnegie & Cabaca, 1991, 1993) in several species. It has been suggested that, upon secretion, TINAGL1 immediately binds to the ECM proximal to the secreting cells or their cell surface receptors, and promotes the adhesion of adrenocortical cells in an autocrine or paracrine manner through interaction with cell surface integrin receptors (Li *et al.*, 2007). In addition, TINAGL1 immobilized on a substratum or bound to FN or collagen promoted adhesion and spreading of adrenocortical cells (Li *et al.*, 2007). In our previous report, we showed that another structural matrix protein, LN1, was not distributed at the blastocoelic surface of the TE and did not colocalize with TINAGL1 at 23:00 h on day 4 of pregnancy (Igarashi *et al.*, 2009). Taken all together, it is conceivable that, just prior to implantation, TINAGL1 secreted from the TE may be involved in some roles of TE-generated BM composed of FN and ColIV, but not LN1, at the blastocoelic surface.

One of the major problems with IVF today is the low pregnancy rate after successful embryo transfer due to implantation failure or early embryonic loss. The fact that growth factor-soaked beads transferred into the uterus of pseudopregnant mice efficiently elicited discrete local implantation-like responses, such as increased vascular permeability, decidualization, and expression of implantation marker genes (Paria *et al.*, 2001), indicates that the low pregnancy rate is attributed to the embryos rather than the uterus. Furthermore, different gene expression patterns between *in vivo* and IVF embryos have been observed (Rinaudo & Schultz, 2004; Rinaudo *et al.*, 2006). In this study, the distributions of TINAGL1 and FN were the same in IVF blastocysts and *in vivo* blastocysts, except that their expressions were low in IVF blastocysts. These data are in accordance with a previous report showing that *in vivo*-derived bovine blastocysts exhibited a 2.64-fold increase in FN expression compared with IVF blastocysts (Mohan *et al.*, 2004). Moreover, in the mouse, it was reported that the expression of *procollagen, type IV alpha 1* mRNAs was 0.60-fold lower in IVF blastocysts than in *in vivo* blastocysts (Giritharan *et al.*, 2007), and immunostaining for ColIV showed that IVF blastocysts contained poorly developed ECM (Summers *et al.*, 2000).

From this study, the following hypothesis is inferred. Low expression levels of ECM proteins, such as FN and ColIV, and supporting molecules, which may