Possible involvement of IFNT in lymphangiogenesis in the corpus luteum during the maternal recognition period in the cow

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Abstract

The corpus luteum (CL), which secretes large amounts of progesterone and is thus essential for establishing pregnancy, contains various types of immune cells that may play essential roles in CL function by generating immune responses. The lymphatic system is the second circulation system and is necessary for immune function, but the lymphatic system of the bovine CL has not been characterized in detail. We collected bovine CLs on days 12 and 16 of the estrous cycle (C12 and C16) and days 16 and 40 of early pregnancy (P16 and P40). Lymphatic endothelial hyaluronan receptor 1 (LYVE1) protein was detected in the CL by immunohistochemistry and western blotting and increased at P40 compared with C16. The mRNA expression levels of lymphangiogenic factors, such as vascular endothelial growth factor-C (VEGFC), VEGFD, and their common receptor VEGFR3, as well as the lymphatic endothelial cell (LyEC) marker podoplanin, increased in P16 and P40 CLs. Thus, it is suggested that the lymphatic system of the bovine CL reconstitutes during early pregnancy. Interferon tau (IFNT) from the conceptus in the uterus is a candidate for activating luteal lymphangiogenesis during the maternal recognition period (MRP). We found that treatment of LyECs isolated from internal iliac lymphatic vessels with IFNT stimulated LyEC proliferation and significantly increased mRNA expression of VEGFC and IFN-stimulated gene 15. Moreover, both IFNT and VEGFC induced LyECs to form capillary-like tubes *in vitro*. In conclusion, it is suggested that new lymphangiogenesis in the bovine CL begins during the MRP and that IFNT may mediate this novel phenomenon.

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Introduction

The corpus luteum (CL) is the essential organ for maintaining pregnancy and accomplishes this by secreting progesterone (P₄). In the cow, the luteal phase of the estrous cycle lasts ~17–18 days, and luteal regression is induced by uterine prostaglandin F_{2α} (PGF_{2α}) in the absence of pregnancy. By contrast, when pregnancy is established, interferon tau (IFNT), a known pregnancy recognition signal in ruminants (Imakawa *et al.* 1987), is secreted by embryonic trophoblast cells and indirectly maintains the CL by attenuating (cow; Meyer *et al.* 1995) or altering (ewe; Zarco *et al.* 1988a, 1988b) luteolytic pulses of uterine PGF_{2α} beginning at approximately day 16 post-insemination; this period is therefore defined as the maternal recognition period (MRP; Spencer *et al.* 2004).

IFNT produced by the conceptus passes through the uterine lumen and enters the endometrium where it also apparently enters venous drainage from the uterus.

Oliveira *et al.* (2008) demonstrated greater antiviral activity in uterine vein blood from day 15 pregnant sheep. PreadSORption of IFNT with antiserum against recombinant ovine IFNT revealed that antiviral activity in uterine vein blood from pregnant ewes was mediated by IFNT (Bott *et al.* 2010). Both of these studies are interpreted to reflect the release of IFNT into the uterine vein. Interestingly, IFN-stimulated gene (ISG) 15 was upregulated in the CL and blood cells as well as in the endometrium during MRP in the pregnant cow and ewe (Hansen *et al.* 1997, Johnson *et al.* 1998, 1999, Han *et al.* 2006, Gifford *et al.* 2007, Yang *et al.* 2010). Furthermore, IFNT enhanced CL resistance to the luteolytic effect of PGF_{2α} in an endocrine manner, and infusion of IFNT into the uterine vein clearly inhibited spontaneous luteolysis in ewes (Bott *et al.* 2010). Therefore, IFNT may influence not only the uterine environment but also the CL in the cow via local or peripheral circulation.

The lymphatic vascular system is considered as the body's second circulation system and is essential for maintaining interstitial fluid pressure equilibrium and transporting tissue fluid, proteins, and cells (Wang & Oliver 2010). The lymphatic system is also crucial during the immune response to infectious agents, as lymphatic vessels are the route by which dendritic cells and macrophages migrate to the lymph nodes and lymphoid organs to present antigen to T cells. While the development of blood vessels, or angiogenesis, has been studied extensively since 1980s, the development of lymphatic vessels, or lymphangiogenesis, has attracted relatively little attention despite the clinical relevance of lymphangiogenesis to processes such as lymphedema and the metastasis of cancer cells. However, study of the lymphatic system is now accelerating due to great progress in identifying specific markers of lymphatic endothelial cells (LyECs), including lymphatic vessel endothelial hyaluronan receptor (LYVE1) and podoplanin (Banerji *et al.* 1999, Breiteneder-Geleff *et al.* 1999, Prevo *et al.* 2001). Importantly, two vascular endothelial growth factor (VEGF) family members, VEGFC and VEGFD, regulate the LyECs via their receptor VEGFR3 and thereby stimulate lymphangiogenesis (Joukov *et al.* 1997, Yamada *et al.* 1997, Karkkainen *et al.* 2004).

The vascular system is well known to be important for the function of the CL (Fraser *et al.* 2000, Wulff *et al.* 2001, Yamashita *et al.* 2008). The development of the bovine CL is accompanied by active angiogenesis, and the structure of blood vessels is retained during pregnancy (O'Shea *et al.* 1989, Lei *et al.* 1991, Beindorff *et al.* 2010). Leukocytes, including monocytes/macrophages, granulocytes, and T lymphocytes, are localized to the CL in several species (Standaert *et al.* 1991, Brannstrom *et al.* 1994, Reibiger & Spanel-Borowski 2000). These findings suggest that the bovine CL should have a lymphatic system with essential roles in regulating the functions of its immune cells. Indeed, early morphological studies revealed profuse networks of lymphatic vessels in the CLs of the sheep, swine, dog, and rabbit (Andersen 1926, Czeizel & Palkovich 1962, Morris & Sass 1966, Murata 1976, Otsuki *et al.* 1987). Moreover, Xu & Stouffer (2009a, 2009b) reported that the VEGFC/VEGFD-VEGFR3 system regulates lymphangiogenesis in and luteal structure and function of the primate CL.

Thus, this study was designed to test the hypothesis that a lymphatic network exists in the bovine CL and the occurrence of lymphangiogenesis changes in the bovine CL during the estrous cycle and early pregnancy. Our novel finding that lymphangiogenesis is upregulated in the CL of pregnancy during MRP led us to investigate the possibility that IFNT affects lymphangiogenesis using LyECs *in vitro*.

Results

LYVE1 protein and mRNA expression in the CL during the estrous cycle and early pregnancy

Lymphatic vessels in bovine tissues were identified by immunohistochemical staining for LYVE1 (a marker of LyECs), and the localization of LYVE1-positive cells was compared with that of von Willebrand factor (VWF, a marker of the vascular endothelial cells)-positive cells (Fig. 1A–H). Serial sections stained for LYVE1 and VWF are shown in Fig. 1A–H. VWF-positive cells were presented (Fig. 1A, black arrow heads) in the lymph node, but the same blood vessels were not stained with LYVE1 (Fig. 1B, white arrow heads). In addition, some cells in the lymph node stained strongly with LYVE1 (Fig. 1B, black arrows) but not with VWF (Fig. 1A, white arrows), suggesting that LYVE1 staining is specific for lymphatic vessels in the cow.

In the mid-luteal phase, the blood vessels of the CL showed appreciable staining for VWF (Fig. 1C, black arrow heads), but the same blood vessels did not stain for LYVE1 (Fig. 1D, white arrow heads). In contrast, LYVE1-positive cells (Fig. 1D, black arrows) were clearly detected in other areas where VWF-positive cells (Fig. 1C, white arrows) were absent. LYVE1 (Fig. 1F, black arrows) and VWF (Fig. 1E, white arrows) staining in the CL remained discrete during pregnancy. The enlarged images in Fig. 1G and H show that LYVE1-positive cells existed in different vessels from VWF-positive cells. LYVE1 expression in the CL during pregnancy was also demonstrated by immunofluorescence (Fig. 1I). Intensity of the LYVE1-staining area was significantly increased from the early to the mid-luteal phase and decreased to regressing luteal phase (Fig. 1J, $P < 0.05$).

Figure 1K–N shows *LYVE1* mRNA and protein expression in the CL during the estrous cycle and early pregnancy. During the estrous cycle, *LYVE1* mRNA expression increased significantly and LYVE1 protein expression tended to increase from the early to the mid-luteal CL (Fig. 1K and L, $P < 0.05$). During early pregnancy, *LYVE1* mRNA levels were greater in the center of the CL at P16 and P40 and in the periphery at P40 (Fig. 1M, $P < 0.05$) than on days 12 and 16 of the estrous cycle. LYVE1 protein levels were also greater ($P < 0.05$) in the periphery of the CL at P40 but not in the center of the CL than on days 12 and 16 of the estrous cycle (Fig. 1N).

Bovine CL is heterogeneous tissue in which there are many arteriolo-venous vessels distributed in the periphery of the CL compared with the center during the mid-luteal phase, while capillary vessels localized at the center more than the periphery of the CL (Shirasuna *et al.* 2009). Therefore, we separated the center and periphery of the CL and investigated different expression of the lymphatic vessels, especially about the CL of pregnancy. It is possible that lymphatic vessels exist both in the periphery and in the center of the bovine CL.

Figure 1 Localization of lymphatic and blood vessels and expression of *LYVE1* mRNA and protein in the bovine corpus luteum. Immunohistochemical localization of VWF and LYVE1 in serial sections of the lymph node (A and B), the CL during the estrous cycle (C and D), the CL during pregnancy (day 100) (E and F), and enlarged images of E and F (G and H). The blood vessels stained by VWF (A, C, E, and G, black arrow heads) were not stained by LYVE1 (B, D, F, and H, white arrow heads), while the lymphatic vessels stained by LYVE1 (B, D, F, and H, black arrows) were not stained by VWF (A, C, E, and G, white arrows). The different staining between VWF and LYVE1 in the tissue sections suggests that lymphatic vessels exist in the bovine CL. LYVE1-positive cells were also detected by immunofluorescent staining (I). The positive staining area of LYVE1 increased in the CL from early to mid-luteal phase (J, $n=3$ /stage). Small black squares in each figure indicate negative control. The scale bars in all images represent 100 μ m. *LYVE1* mRNA expression (K) and protein expression (L) in the bovine CL during the estrous cycle ($n=4-5$ /stage). *LYVE1* mRNA (M) and protein (N) expression in the bovine CL at days 12 (C12) and 16 (C16) of the estrous cycle and at days 16 (P16) and 40 (P40) of pregnancy ($n=5-7$ /stage). Black bars indicate the peripheral area of the CL (M and N), while white bars indicate the central area. Representative western blots are shown for *LYVE1* (42 kDa) and β -actin (37 kDa) (L and N). All values are shown as mean $\pm$ s.e.m. Different superscript letters (a and b; periphery of the CL, x and y; center of the CL) indicate significant differences ($P<0.05$) and # indicates tendency to difference ($P<0.1$) as determined by ANOVA followed by Fisher's multiple comparison test.

Figure 2 mRNA expression of *VEGFC*, *VEGFD*, *VEGFR3*, and podoplanin in the bovine CL during the estrous cycle. mRNA expression of *VEGFC* (A), *VEGFD* (B), *VEGFR3* (C), and podoplanin (D) increased in the CL from the early to the mid-luteal phase and decreased to regressing luteal phase (mean $\pm$ S.E.M., $n=4-5$ /stage). Different superscript letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by Fisher's multiple comparison test.

because of the expression of LYVE1 protein and mRNA in both area of the CL. LYVE1 protein expression was greater in the periphery of the CL of P40 but not in the center (Fig. 1 N), indicating the possibility that lymphatic vessels are remodeled especially in the periphery of the CL during pregnancy.

The mRNA expression of lymphangiogenic factors and a LyEC marker during the estrous cycle and early pregnancy

The mRNA expression levels of lymphangiogenic factors (*VEGFC*, *VEGFD*, and *VEGFR3*) and a LyEC marker (podoplanin) in the CL during the estrous cycle are shown in Fig. 2. The mRNA expression of all factors significantly increased ($P<0.05$) from the early to the mid-luteal phase (Fig. 2A–E). The mRNA expression levels of *VEGFC*, *VEGFD*, and podoplanin decreased ($P<0.05$) during luteal regression (Fig. 2A, C, and D).

The mRNA expression levels of *VEGFC*, *VEGFD*, and *VEGFR3* and podoplanin in the CL during early pregnancy are shown in Fig. 3. *VEGFC* mRNA expression was higher in the peripheral CL at P16 and in the central CL at P40 compared with C16 (Fig. 3A, $P<0.05$). *VEGFD* and *VEGFR3* mRNA levels were higher in the peripheral and in the central CL at P16 compared with C16. Although *VEGFD* mRNA expression in the CL decreased from P16 to P40, *VEGFR3* mRNA in the peripheral CL was maintained at a higher level at P40 compared with P16 (Fig. 3B and C, $P<0.05$). In addition, podoplanin mRNA expression was significantly higher in the peripheral CL at P16 compared with C16 and further increased in the peripheral and central CL at P40 compared with P16 (Fig. 3D, $P<0.05$).

Characteristics of LyECs compared with luteal endothelial cells

To investigate the purity of LyECs, the mRNA and protein expression levels of lymphatic markers were compared between isolated LyECs and luteal endothelial cells (LECs; Fig. 4). LyECs but not LECs strongly expressed podoplanin (LyEC marker) mRNA (Fig. 4A, $P<0.01$). Figure 4B shows light field of LyECs. By immunofluorescence, LyECs were stained with LYVE1 antibody (Fig. 4C), while LECs did not express LYVE1 protein (Fig. 4D). LyECs stained without primary antibody, as a negative control did not show LYVE1 staining (Fig. 4E). In contrast, previous studies showed that same LECs were stained with VWF (Klipper *et al.* 2004), but VWF protein did not express in LyECs in this study (data not shown). These results indicated that isolated LyECs had specific characteristics of LyECs.

The mRNA expression levels of lymphangiogenic factors in LyECs compared with LECs, luteal cells, and immune cells

Immune cells have an important role in lymphangiogenesis because inflammatory cells such as monocytes/macrophage can secrete VEGFC and VEGFD, inducing inflammatory lymphangiogenesis (Cursiefen *et al.* 2004, Baluk *et al.* 2005, Iwata *et al.* 2007). We determined the mRNA expression levels of lymphatic-related factors in peripheral blood mononuclear cells (PBMCs), polymorphonuclear neutrophils (PMNs), luteal cells (LCs), LECs, and LyECs (Fig. 5). LyECs, LECs, and LCs expressed

Figure 3 mRNA expression of *VEGFC*, *VEGFD*, *VEGFR3*, and podoplanin in the bovine CL during early pregnancy. mRNA expression of *VEGFC* (A), *VEGFD* (B), *VEGFR3* (C), and podoplanin (D) increased in the CL of day 16 (P16) or day 40 (P40) during pregnancy compared with day 12 (C12) and/or day 16 (C16) during the estrous cycle (mean $\pm$ S.E.M., $n=5-7$ /stage). Black bars indicate the periphery area of the CL, while white bars indicate the central area. Different superscript letters (a and b; periphery of the CL, x and y; center of the CL) indicate significant differences ($P<0.05$) as determined by ANOVA followed by Fisher's multiple comparison test.

Figure 4 Characteristics of LyECs compared with LECs. Podoplanin mRNA was expressed mainly in LyECs but not in LECs (A) (mean $\pm$ s.e.m., $n=5$ /group). B shows the light field of LyECs. LYVE1 protein was detected only in LyECs (C) but not in LECs (D) using immunofluorescence. E shows a negative control for LYVE1. The scale bars represent 50 μ m. **Significant differences ($P<0.01$) as determined by Student's *t*-test.

VEGFC mRNA (Fig. 5A, $P<0.05$), and expression levels of *VEGFC* mRNA were higher in LyECs than in LECs and LCs (Fig. 5A, $P<0.05$). PBMCs and PMNs did not express *VEGFC* mRNA (Fig. 5A, $P<0.05$). In contrast, *VEGFD* mRNA was expressed in PBMCs and PMNs but not in LCs, LECs, or LyECs (Fig. 5B). *VEGFR3* mRNA, which is a marker of LyECs, was expressed only in LyECs (Fig. 5C).

Effects of IFNT or IFNA on *ISG15*, *VEGFC*, and *VEGFR3* mRNA expression in LyECs and LECs

We investigated whether IFNT has the specific effects for LyECs compared with IFN α (IFNA), which belongs to type 1 IFN as well as IFNT. The changes in *ISG15*, *VEGFC*, and *VEGFR3* mRNA expression levels in LyECs and LECs after treatment with IFNT or IFNA (0.6, 6, or 60 IU/ml) are shown in Fig. 6. IFNT increased *ISG15* mRNA expression in a dose-dependent manner in both LyECs and LECs (Fig. 6A and D). In addition, IFNT at 60 IU/ml stimulated *VEGFC* mRNA expression in LyECs (Fig. 6B, $P<0.05$) but had no effect on *VEGFR3* mRNA expression (Fig. 6C). In LECs, *VEGFC* mRNA levels tended to increase on treatment with IFNT at 60 IU/ml (Fig. 6E, $P<0.1$). IFNA also stimulated *ISG15* expression in LyECs at 6 IU/ml and in LECs at 60 IU/ml (Fig. 6F and I, $P<0.05$). However, IFNA at 0.6 IU/ml decreased *VEGFC* mRNA expression in LyECs (Fig. 6G, $P<0.1$) and had no effect in LECs (Fig. 6J). IFNA also had no effect on *VEGFR3* mRNA expression in LECs (Fig. 6H).

Effects of IFNT on proliferation of LyECs and capillary-like tube formation

We next examined the effects of IFNT on the proliferation of LyECs and capillary-like tube formation of LyECs

in vitro (Fig. 7A–E). The data show that IFNT at 6 IU/ml stimulated proliferation of LyECs compared with the control after 24 h (Fig. 7A, $P<0.05$). We also assessed capillary-like tube formation in response to IFNT. In an *in vitro* matrigel assay, both IFNT (60 IU/ml) and *VEGFC* (100 ng/ml) stimulated LyECs to assemble into capillary-like structures (Fig. 7B–E, $P<0.01$). Lower concentration of IFNT (6 IU/ml) tends to promote capillary-like structures of LyECs (Fig. 7B, $P<0.1$).

Discussion

In this study, we have performed a detailed analysis of the lymphatic system of the bovine CL during the estrous cycle and early pregnancy. The mRNA expression levels of LyEC markers and lymphangiogenic factors were markedly higher in the CL during the MRP than during the estrous cycle. Therefore, we hypothesized that IFNT produced by trophoblast cells of the developing conceptus during the MRP stimulates the CL to upregulate the mRNA expression of lymphatic-related factors. In fact, IFNT increased *VEGFC* mRNA expression in LyECs and promoted the proliferation and tube formation of LyECs *in vitro*. Although the effects of IFNT on CL-derived LyECs remain to be investigated, the present results suggest the possibility that IFNT regulates lymphangiogenesis in the early pregnant CL during the MRP.

I.u. administration of IFNT has been shown to inhibit the release of PGF $_{2\alpha}$ due to suppression of oxytocin receptor (OXTR) expression in the endometrium (Spencer & Bazer 1996, Chen *et al.* 2006). In addition, OXTR is regulated primarily by receptors for estrogen receptor α (ESR1; Spencer & Bazer 1996, Spencer *et al.* 2004).

Figure 5 The mRNA expression of lymphangiogenic factors in LyECs compared with LECs, luteal cells, and immune cells. *VEGFC* mRNA was detected mainly in LCs, LECs, and LyECs but not in PBMCs and PMNs (A). In contrast, PBMCs and PMNs expressed *VEGFD* mRNA (B). *VEGFR3* mRNA was expressed only in LyECs (C). All values are shown as mean $\pm$ S.E.M. ($n=5$ /group). Different superscript letters indicate significant differences ($P<0.05$) as determined by ANOVA followed by Fisher's multiple comparison test.

While ESR1 is up-regulated during the estrous cycle, that upregulation is blocked by IFNT in pregnant ewes (Spencer *et al.* 1995, Fleming *et al.* 2006). A s.c. injection of IFNT had no effect on the expression of ER and OXTR in the ewe, suggesting that IFNT acts only within the uterus (Spencer *et al.* 1999). However, IFNT produced by the conceptus was recently shown to pass through the uterine lumen and enter the uterine vein (Oliveira *et al.* 2008, Bott *et al.* 2010). In addition, *ISG15* mRNA expression in both the endometrium and the CL is upregulated during MRP in the pregnant ewe and cow compared with the cyclic animal (Hansen *et al.* 1997, Johnson *et al.* 1998, 1999, Yang *et al.* 2010). *ISG15* mRNA were highly expressed in endometrium and CL, but not in liver, from day 15 pregnant ewes compared with nonpregnant ewes, suggesting some degree of countercurrent exchange of IFNT from the uterine vein to the ovarian artery during pregnancy (Bott *et al.* 2010). It has been proposed that IFNT may have endocrine action through inducing CL resistance to PGF_{2 α} and long-term survival of the CL and maintenance of pregnancy (Hansen *et al.* 2010). The mRNA expression of *IFNT* by the bovine conceptus peaked on day 16 of pregnancy (Farin *et al.* 1990), the same time at which the increased expression of lymphangiogenic factors such as *LYVE-1*, *VEGFC*, *VEGFD*, and *VEGFR3* was evident in this study (Figs 1M, N and 3), suggesting that the upregulation of lymphatic-related factors in the bovine CL during the MRP may be related to IFNT.

Bovine CL consists of various cell types including LCs, vascular endothelial cells, smooth muscle cells, fibroblasts, and immune cells (O'Shea *et al.* 1989, Lei *et al.* 1991, Zheng *et al.* 1993, Penny 2000). Therefore, we first examined the mRNA expression of the lymphangiogenic factors in various cells, including LCs and LECs, isolated from the CL, LyECs isolated from the internal iliac lymphatic vessels, and PBMCs and PMNs isolated from peripheral blood (Fig. 5). LyECs, LECs, and LCs have a potential of producing VEGFC. In contrast, immune cells such as PMN and PBMC have a potential of producing VEGFD. It is therefore possible that several cell types in the bovine CL can induce lymphangiogenesis.

VEGFC is recognized to be more potent than VEGFD for activating lymphangiogenesis. Deletion of *VEGFC* leads to complete absence of the lymphatic vasculature and death in mouse embryos (Karkkainen *et al.* 2004). By contrast, while deletion of *VEGFD* does not affect the development of lymphatic vessels, exogenous VEGFD protein rescues the impaired vessel sprouting of *VEGFC*^{-/-} embryos (Karkkainen *et al.* 2004, Baldwin *et al.* 2005). Therefore, we focused on LyECs and LECs, both of which abundantly expressed *VEGFC* mRNA and could thus contribute to lymphangiogenesis. In particular, we examined the hypothesis that IFNT is related to the upregulation of VEGFC in the CL during the MRP. As expected, IFNT stimulated the mRNA expression of not only *ISG15* but also *VEGFC* in both LyECs and LECs (Fig. 6). Although VEGFC stimulated LyECs to form tubes, as in previous studies (Podgrabska *et al.* 2002, Zeng *et al.* 2006), IFNT promoted comparable levels of LyEC proliferation and capillary-like tube formation (Fig. 7). The data indicate that IFNT can induce lymphangiogenesis in cows by stimulating *VEGFC* expression in both LyECs and LECs, suggesting that IFNT might be responsible for the increase of lymphangiogenic factors in the bovine CL during early pregnancy.

IFNs are typically classified as either type 1 or type 2. Type 1 IFNs comprise IFNA, IFN β , and IFNT and defense against infection by pathogens is considered to be the main function of this group (Roberts *et al.* 1996, Spencer *et al.* 2004). IFNT has sequence similarity ($\sim 50\%$ amino acid identity) and functional homology to IFNA (Roberts *et al.* 1992). All type 1 IFNs bind to common receptors such as IFNA receptor 1 (IFNA R1) and IFNA R2 (Li & Roberts 1994) and induce several common genes (Teixeira *et al.* 1997, Staggs *et al.* 1998, Hansen *et al.* 1999, Asselin *et al.* 2001, Choi *et al.* 2001). It is therefore possible that the IFNT-induced lymphangiogenesis shown in this study is not specific and that IFNA can also activate lymphangiogenesis. In contrast with the effect of IFNT on LyECs, IFNA tended to decrease *VEGFC* mRNA expression in LyECs despite stimulating *ISG15* mRNA expression. Although IFNT increased mRNA expression of both *VEGFC* and *ISG15* in LECs, IFNA upregulated *ISG15* mRNA expression but

Figure 6 Effects of IFNT or IFNA on *ISG15*, *VEGFC*, and *VEGFR3* mRNA expression in LyECs and LECs. IFNT stimulated mRNA expression of *ISG15* and *VEGFC* but not *VEGFR3* in LyECs (A–C). IFNT also stimulated *ISG15* and *VEGFC* mRNA expression in LECs (D and E). In contrast, IFNA stimulated *ISG15* mRNA expression (F) but tended to decrease *VEGFC* mRNA expression in LyECs (G). IFNA had no effect on *VEGFR3* mRNA expression (H). In LECs, IFNA stimulated *ISG15* mRNA expression but not *VEGFC* mRNA expression (I and J). White bars indicate the control group (no treatment), while black bars indicate 0.6–60 IU/ml IFNT-treated or IFNA-treated groups. All values are shown as mean $\pm$ S.E.M. ($n=4$ /group). *Significant differences ($P<0.05$) and #tendency to difference ($P<0.1$) as determined by ANOVA followed by Fisher's multiple comparison test.

not *VEGFC* mRNA expression in LECs. In fact, Green *et al.* (2005) reported different effects of IFNT and IFNA in the ewe, with IFNT treatment resulting in enhanced P_4 production without luteolysis while treatment with IFNA did not affect CL function and allowed normal regression of the CL. Moreover, Nagaoka *et al.* (2003) also showed a specific effect of IFNT distinct from that of IFNA. While both IFNT and IFNA stimulated the expression of IFN γ -inducible protein 10 kDa (IP-10) by monocytes, the expression of *IP-10* mRNA by endometrial explants *in vitro* was much more effectively stimulated by IFNT (Nagaoka *et al.* 2003).

The mRNA and protein expression levels of the lymphangiogenic factors and a marker of lymphatic vessels in the bovine CL changed significantly between the early and the mid-luteal phases of the estrous cycle (Figs 1J, K, L and 2). In agreement with our results, Xu & Stouffer (2009a, 2009b) revealed that lymphatic vessels are present in the primate CL and that mRNA levels of

VEGFC, *VEGFD*, and *VEGFR3* changed throughout the menstrual cycle. Furthermore, an injection of soluble VEGFR3 (which acts as an anti-VEGFR3 antibody) into the preovulatory follicle inhibited follicle rupture/ovulation and suppressed P_4 production in the monkey CL (Xu & Stouffer 2009a, 2009b). In addition, the bovine CL is known to be a highly vascularized organ, and its development and regression are regulated locally by many angiogenic and vasoactive factors (Girsh *et al.* 1996, Skarzynski *et al.* 2000, Miyamoto *et al.* 2009, 2010, Robinson *et al.* 2009). The mRNA expression of *VEGFA* and fibroblast growth factor-2 (*FGF2*), which are the major factors regulating angiogenesis throughout the luteal phase, is highest immediately after ovulation (Berisha *et al.* 2000, Schams & Berisha 2004, Woad *et al.* 2009). Interestingly, both *VEGFA* and *FGF2* have potent lymphangiogenic activity (Kubo *et al.* 2002, Cursiefen *et al.* 2004). *VEGFC* can stimulate both lymphangiogenesis (via *VEGFR3*) and angiogenesis

Figure 7 Effects of IFNT on proliferation of LyECs and capillary-like tube formation. IFNT stimulated the proliferation of LyECs (A) and promoted the capillary-like tube formation of LyECs as well as 100 ng/ml VEGFC (B). Panels C, D and E show typical images of capillary-like tube formation in Matrigel assays on LyECs. All values are shown as mean $\pm$ s.e.m. ($n=4$ /group). The scale bars represent 500 μ m. *, **Significant differences ($P < 0.05$ or $P < 0.01$) and #tendency to difference ($P < 0.1$) as determined by ANOVA followed by Fisher's multiple comparison test.

(via VEGFR2; Millauer *et al.* 1993, Shalaby *et al.* 1995, Wilting *et al.* 1997). Therefore, multiple vascular growth factors may induce lymphangiogenesis and angiogenesis in the developing bovine CL. Lymphatic vessels also appear to degenerate with blood vessels during luteal regression. This concept is supported by a previous report that mRNA expression levels of *VEGFC*, *VEGFD*, and *LYVE1* were reduced in the bovine CL after $\text{PGF}_{2\alpha}$ injection (Schams *et al.* 2009).

In summary, the present results demonstrate the existence of lymphatic vessels in the bovine CL and provide evidence that the increase in lymphangiogenic factors in the CL in the cow begins during the MRP. Importantly, IFNT has the potential to promote lymphangiogenesis by increasing VEGFC, which is also likely present in the bovine CL during the MRP.

Materials and Methods

CLs were collected at the Clinic for Cattle, University of Veterinary Medicine Hannover, Germany, and blood was collected at the Field Center of Animal Science and Agriculture, Obihiro University. Each experimental procedure complied with the ethics committee on animal rights protection, Oldenburg, Germany, in accordance with German legislation on animal rights and welfare (file reference number 33.9-42502-04-07/1275) and the Guidelines for the Care and Use of Agricultural Animals of Obihiro University.

Reagents

DMEM/nutrient mixture and Ham's F-12 medium (1:1), PBS, amphotericin B, gentamicin, streptomycin and penicillin G solution, collagenase from clostridium histolyticum type IV, Tween 20, anti- β -actin mouse monoclonal (clone AC-15 antibody), ECL Western Blotting Detection System, and 0.3%

H_2O_2 were purchased from Sigma; BSA was purchased from Wako (Osaka, Japan); TRIzol reagent and rabbit anti-rabbit conjugated to Alexa 568 IgG were purchased from Invitrogen Corporation; tissue and cell culture plates (48- and 24-wells/plate) were purchased from Nunc (Roskilde, Denmark); optimal cutting temperature (OCT) compound was purchased from Sakura Finetechnical (Tokyo, Japan); GNRH was purchased from Intervet (Unterschleißheim, Germany); DNase using a commercial kit was purchased from SV total RNA Isolation System: Promega Co.; THE RNA storage Solution was purchased from Ambion, Inc. (Austin, TX, USA); DNA purification kit SUPRECTM-01 was purchased from TaKaRa Bio. Inc. (Otsu, Japan); 4% Block Ace Powder was purchased from DS Pharma Biomedical (Osaka, Japan); anti-mouse-LYVE1 rabbit polyclonal antibody was purchased from Abcam (Cambridge, UK); BD Matrigel basement membrane was purchased from BD Biosciences (Bedford, MA, USA); recombinant human VEGFC was purchased from ProSci (Poway, CA, USA); protease inhibitor cocktail was purchased from Roche; 70 μ m filter (Cell Strainer, REF 352350) was purchased from BD Falcon (Franklin Lakes, NJ, USA); PVDF membranes were purchased from Bio-Rad Laboratories; lymphoprep was purchased from Axis-Shield (Oslo, Norway); HRP-conjugated anti-rabbit IgG antibodies were purchased from GE Healthcare Ltd (Chalfont St Giles, UK); HRP-conjugated anti-mouse IgG antibodies were purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA); anti-human VWF rabbit polyclonal antibody (clone A0082) was purchased from Dako Denmark A/S (Glostrup, Denmark); biotinylated goat anti-rabbit BA-1000 IgG and avidin-biotin reagent (PK-6100, Vectastain ABC kit) were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA); recombinant bovine IFN was produced by *Escherichia coli*. Briefly, bovine IFN cDNA (bTP-509A, gifted by Dr RM Roberts, University of Missouri) was inserted into pET-21a (Invitrogen) *E. coli* expression vector. This vector was then introduced into the BL21pLys (DE3) strain (Invitrogen) and the expression of the gene was induced by IPTG (Sigma). The

produced protein was deposited in inclusion body; therefore, it was solubilized by denature and renature processes. After purification of the crude IFN by HPLC, its activity as determined in a viral resistance assay using bovine kidney MDBK cells was found to be 59 050 IU/ml (5.95×10^5 IU/mg and 456 μ M) and evaluated by prolongation of CL life span following its infusion into the uterine horn; recombinant bovine IFN was purchased from Kingfisher Biotech (St Paul, MN, USA). The specific activity as determined in avian resistance assay using bovine kidney MDBK cells was found to be 9000 IU/ml.

Collection of the bovine CLs

CLs during the estrous cycle and pregnancy

Ovaries were collected from a local slaughterhouse in Japan and CLs were classified according to stage of the estrous cycle by macroscopic observation as described previously (size, consistency, connective tissue, and mucus; Miyamoto & Schams 1991). The stages of the estrous cycle were estimated as follows: early (days 3–5), mid (days 8–12), late (days 13–15), and regression (day 18<) of the estrous cycle ($n=4-5$ in each stage). Additionally, CLs were obtained from pregnant cows that were confirmed to have fetus (estimated fetal ages from fetal crown-rump length for ~ 100 days of pregnancy).

CLs during early pregnancy

Seven normal cyclic German Holstein cows were used as in previous *in vivo* study in Germany (Beindorff *et al.* 2010). Briefly, the animals were divided into four groups: ovariectomy was performed on days 12 and 16 (C12 and C16, $n=5$) of the estrous cycle (not inseminated) as well as on days 16 and 40 (P16 and P40, $n=5-7$) of pregnancy. We selected day 16 after AI as the transitional phase to pregnancy (MRP) and day 40 as establishment phase of pregnancy (implantation of embryo), in comparison with the mid and late luteal phase (days 12 and 16) during the estrous cycle. To determine the exact day of ovulation, all cows received GNRH (0.01 mg buserelin, 2.5 ml of receptal), followed 7 days later by PGF_{2 α} , and then received GNRH at 48 h after PGF_{2 α} . Only animals that had a pre-ovulatory follicle by the last GNRH application attended the study. Artificial inseminations were carried out 12 and 24 h after GNRH application. Two days after GNRH, ovulation (=day 1) was approved by ultrasonography in all animals. To check the pregnancy for day 16 inseminated cows, the cows were killed after ovariectomy and we determined the presence of an embryo within the uterus. In this collection, CLs obtained from early pregnant cows were performed in the periphery and the center of the CL separately as in our previous study (Shirasuna *et al.* 2008).

To use immunohistochemical analysis, CLs were fixed with 4% paraformaldehyde and embedded in OCT compound according to make frozen tissue specimens. The CL tissue samples were collected, minced, and then immediately placed into a 1.5 ml microcentrifuge tube with or without 400 μ l TRIzol reagent and stored at -80°C until analysis.

Collection of bovine lymph nodes

The lymph nodes were taken from cattle freshly dissected at Obihiro University. The lymph nodes were temporarily stored in warm PBS and processed within 30 min of collection. For immunohistochemical analysis, lymph nodes were fixed with 4% paraformaldehyde and embedded in OCT compound as for frozen tissue specimens. The remaining lymph nodes were prepared for molecular biology purposes.

Isolation of PBMCs and PMNs

PBMCs and PMNs were isolated from whole blood collected from the jugular vein (Jientaweewoon *et al.* 2011). PBMCs and PMNs were isolated by centrifugation at 1000 *g* for 30 min at 10°C over lymphoprep. After PBMCs were collected, the plasma and buffy coat were discarded to collect the PMN. Hypotonic distilled water was added to the PBMCs and PMNs for ~ 10 s to remove red blood cells. Isotonicity was restored by the addition of twice concentrated PBS and cells were collected by centrifugation at 500 *g* for 10 min at 10°C . This lysis procedure was repeated on the cell pellet two times. Isolated immune cells were stored at -80°C until analysis of mRNA expression.

Isolation and culture of LyECs

Methods for isolation and culture of bovine LyECs were adapted from previously established methods in cows (Leak *et al.* 1999, Nguyen *et al.* 2007). Sections of the internal iliac lymphatic vessels were taken from cattle freshly dissected at Obihiro University. Trypan blue, which is injected into the internal iliac lymph node, outlined the internal iliac lymphatic vessels, but not blood vessels. Trypan blue-stained lymphatic vessels were cut from surrounding fatty tissue and blood vessels that were not filled with trypan blue. The lymphatic vessels were stored temporarily in warm PBS and processed within 30 min of collection. Connective and adipose tissues were removed to expose the lymph nodes and vessels. Trypan blue was flushed from the excised vessels with warm PBS supplemented with 1% amphotericin B and 0.1% gentamicin. The distal ends of excised vessels were ligated with surgical suture, and the vessels were infused with a solution of 1.5 mg/ml collagenase in PBS. The vessels were occluded at both ends to trap the enzyme solution and incubated in PBS at 37°C for 15 min. The released endothelial lining cells were collected, washed seven times with PBS, and plated on 1% Vitrogen pre-coated plates (24-well plates). The cells were cultured in DMEM/F-12 medium containing 5% FBS, 1 M NaHCO₃, gentamicin solution (50 mg/l), and amphotericin B solution (2.5 mg/l) and scratched in order to separate the LyECs from other cells such as fibroblasts. Isolated cells at passage 5–8 were used for the following experiments. Before IFN treatment, LyECs were washed twice with PBS. Cells were incubated in DMEM/F-12 medium containing 0.1% FBS, 1 M NaHCO₃, gentamicin solution (50 mg/l), and amphotericin B solution (2.5 mg/l) for 24 h at 37°C with the following additions: control (no further addition), IFNT (0.6, 6, or 60 IU/ml), or

IFNA (0.6, 6, or 60 IU/ml). Each treatment was performed in duplicate in 24-well plates. At the end of the treatment period, the cells were stored at -80°C until analysis of mRNA expression.

Isolation and culture of LCs and LECs

The CLs of the mid luteal phase were collected at local slaughterhouse and dispersed using collagenase IV. The luteal stages were classified as mid (days 8–12) by macroscopic observation of the ovary as described earlier. LECs were isolated from the CLs as described previously (Spaniel-Borowski 1991). As per the previous study, magnetic tosylactivated beads coating with BS-1 lectin bind glycoproteins on the bovine endothelial cells and can isolate only LECs from mixed cells of the bovine CL tissue. After removing of LECs (BS-1-positive cells), the rest of mixed cells were detected LCs as BS-1 lectin-negative cells (Klipper *et al.* 2004). LECs were cultured in DMEM/F-12 medium containing 5% FBS, 1 M NaHCO_3 , gentamicin solution (50 mg/l), and amphotericin B solution (2.5 mg/l) in 24-well plates. Before IFN treatment, LECs were washed twice with PBS. Cells were incubated in DMEM/F-12 medium containing 0.1% FBS, 1 M NaHCO_3 , gentamicin solution (50 mg/l), and amphotericin B solution (2.5 mg/l) for 24 h at 37°C with the following additions: control (no further addition), IFNT (0.6, 6, or 60 IU/ml), or IFNA (0.6, 6, or 60 IU/ml). At the end of the treatment period, the cells were stored at -80°C until analysis of mRNA expression.

Immunohistochemistry

Serial sections (7 μm thick) of the CLs were mounted on glass microscope slides coated with APS and immunohistochemistry for LYVE1 (a specific marker of LyECs) and VWF (a specific marker of endothelial cells) was performed. Sections were deparaffinized in xylene and rehydrated using decreasing concentrations of ethanol. The sections were incubated in Tris buffer (pH 10) at 98°C for 10 min and immersed in 0.3%

H_2O_2 in methanol for 1 h to block endogenous peroxidase activity. Sections were then rinsed with TBS, incubated with 4% Block Ace in TBS to reduce nonspecific reactions and incubated with polyclonal antibody against LYVE1 (diluted 1:200) or VWF (diluted 1:200) at 4°C overnight. As a negative control, the sections were incubated with goat anti-rabbit IgG overnight at 4°C . Thereafter, sections were rinsed three times for 5 min in TBS and incubated with biotinylated goat anti-rabbit IgG (1:400) at room temperature for 1 h. Sections were then incubated with avidin–biotin reagent (1:2) for 30 min, and positive signals were visualized using 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in 50 mM Tris–HCl (pH 7.4) containing 0.02% H_2O_2 followed by nuclear staining with hematoxylin.

Immunofluorescence analysis of LyECs and the CL during the estrous cycle

The experimental method was modified from that of Maliba *et al.* (2008). LyECs were grown to confluence, rinsed twice with PBS, and fixed with 4% paraformaldehyde–PBS solution for 10 min at room temperature. Following fixation, the cells were washed three times with PBS and blocked with 4% Block Ace Powder in Tris-buffered saline (TBS) for 15 min at room temperature. Cells were then incubated with rabbit anti-mouse-LYVE1 polyclonal antibody (1:100 dilution) for 90 min at room temperature, rinsed with PBS, and incubated with anti-rabbit IgG conjugated to Alexa 568 (1:400 dilution) for 60 min at room temperature. The cells were observed with a confocal microscope (DMI6000B, Leica Microsystems, USA), and the Alexa 568-conjugated antibody was visualized using a 568 nm argon laser.

The CLs of estrous cycle were stained with LYVE1 antibody as well as the LyECs. After overnight incubation with LYVE1 antibody or goat anti-rabbit IgG as negative control, sections were incubated with anti-rabbit IgG conjugated to Alexa 568 (1:100 dilution) at room temperature for 1 h. The CLs were observed with a confocal microscope, and the Alexa 568-conjugated antibody was visualized using a 568 nm argon laser. The intensity of LYVE1

Table 1 Primer sequences for the investigated genes.

Gene	Primer sequence	Accession no.	Product size (bp)
<i>ISG15</i>	FWD: GGT ATG CGA GCT GAA GCA GTT REV: ACC TCC CTG CTG TCAA GGT	NM_174366	87
<i>LYVE1</i>	FWD: AGG TTG AAG AAG CAC GGA AA REV: AGG GAT CAT CGG TGG TGA TA	NM_205815	231
Podoplanin	FWD: TGG CTA CGG AGC TTT TTC AT REV: CAC ACC CAG GGT TGT TTT CT	ENSBTAT 00000002341	291
<i>VEGFR3</i>	FWD: TGA GGA TAA AGG CAG CAT GGA REV: CCC AGA AAA AGA CAG CGA TGA	AF030379	66
<i>VEGFC</i>	FWD: CTC AAG GCC CCA AAC CAG T REV: CAT CCA GCT TAG ACA TGC ATC G	NM_174488	71
<i>VEGFD</i>	FWD: GGA GAA TGC CTT TTG AAC CA REV: CCA GTC CTC GAA GTG TGT GA	NM_001101043 XM_590821	272
β -actin	FWD: CCA AGG CCA ACC GTG AGA AGA T REV: CCA CGT TCC GTG AGG ATC TTC A	K00622	256
<i>GAPDH</i>	FWD: CTC TCA AGG GCA TTC TAG GC REV: TGA CAA AGT GGT CGT TGA GG	ENSBTAT 00000019604	120

staining was calculated by the pixel sum/area of the LyECs using the accessory software for this microscope.

Proliferation of LyECs

LyECs were grown on plates pre-coated with 1% Vitrogen in complete DMEM/F-12 containing 5% FBS (1×10^5 cells/well in 24-well plates) for 24 h at 37 °C. LyECs were then rinsed twice with PBS and incubated for 24 h in fresh DMEM/F-12 medium containing 0.1% FBS, 1 M NaHCO₃, gentamicin solution (50 mg/l), and amphotericin B solution (2.5 mg/l) supplemented with IFNT (0.6, 6, or 60 IU/ml). After 24 h, LyECs removed from the dishes were centrifuged at 170 g (1000 r.p.m.) for 10 min. After the centrifuge, cell lysate was mixed with the same amount of trypan blue solution. LyEC proliferation was calculated as the number of living LyECs counted by light microscopy (dead cells were stained with trypan blue).

Capillary tube formation on matrigel of LyECs

LyEC capillary tube formation was evaluated as described previously (Yasuda *et al.* 2000). Briefly, 48-well plates were coated with 200 µl/well of BD Matrigel basement membrane at 4 °C and then incubated at 37 °C for at least 1 h to allow polymerization. LyECs (2×10^4 cells/well) were plated in a final volume of 0.5 ml/well of LyEC culture medium containing IFNT (60 IU/ml) or recombinant human VEGFC (100 ng/ml). After incubation for 8 h, tube formation was examined visually and three randomly chosen images taken by inverted microscopy at a magnification of $\times 100$ were imported and the total length of tube formation in each image (1 $\times$ 1 mm) was analyzed. Data are shown as mean $\pm$ S.E.M.

RNA extraction, cDNA synthesis, and RT quantitative PCR

Total RNA was extracted from CL, PBMC, PMN, LyECs, LECs, and LCs following the protocol of Chomczynski & Sacchi (1987) using TRIzol reagent, treated with DNase using a commercial kit, and frozen at -20 °C in THE RNA Storage Solution. The cDNA was synthesized as described previously (Watanabe *et al.* 2006). The mRNA expression levels of *ISG15*, *LYVE1*, podoplanin, *VEGFR3*, *VEGFC*, *VEGFD*, β -actin, and *GAPDH* were quantified by RT quantitative PCR (RT-qPCR) as described previously (Watanabe *et al.* 2006). RT-qPCRs were performed in duplicate in a final volume of 10 µl containing 5 µl SYBER Green, 2.8 µl of H₂O, 0.1 µl of 50 µM forward and reverse primers (Table 1 lists primer sequences and accession numbers), and 2 µl cDNA template or water (as nontemplate negative control). RT-qPCR conditions were 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, and 56 °C for 30 s using a LightCycler (Roche Diagnostics Co.). The PCR products were subjected to electrophoresis, and the target bands were cut out and purified using a DNA purification kit (SUPRECTM-01). The mRNA expression levels were normalized using β -actin or GAPDH as an internal standard. Each PCR amplification was sequenced to confirm using an Applied Biosystems 3730X1 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Western blotting

The CL tissue samples were homogenized in lysis buffer containing 25 mM Tris-HCl, pH 7.4, 0.3 M sucrose, 2 mM Na₂EDTA, and protease inhibitor cocktail and then filtered with a 70 µm filter. The proteins were dissolved in sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10% SDS, 0.5% bromophenol blue) and steamed for 5 min. The entire samples were subjected to electrophoresis on 10% SDS-PAGE gels for 50 min at 200 V. The proteins were transferred to PVDF membranes for 2 h at 60 V. The membranes were blocked with 4% Block Ace Powder in TBS with 0.5% Tween-20 for 1 h at room temperature. The membranes were next incubated with a rabbit anti-mouse-LYVE1 polyclonal antibody (1:500 dilution) and a mouse anti- β -actin monoclonal clone AC-15 antibody (1:10 000 dilution). The membranes were then washed three times in TBS with 0.5% Tween-20, incubated with HRP-conjugated anti-rabbit (1:10 000 dilution) or anti-mouse (1:10 000 dilution) IgG antibodies for 1 h at room temperature, and washed three times with TBS with 0.5% Tween-20. The signals were detected using an ECL Western Blotting Detection System. The optical densities of the immunospecific bands were quantified using an NIH image computer-assisted analysis system.

Statistical analysis

All data are presented as mean $\pm$ S.E.M. The statistical significance of differences was assessed by one-way ANOVA followed by Fisher's multiple comparison test or Student's *t*-test. A *P* < 0.05 was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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