Possible action of vasohibin-1 as an inhibitor in the regulation of vascularization of the bovine corpus luteum

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Abstract

The development of the corpus luteum (CL), which secretes large amounts of progesterone to establish pregnancy, is accompanied by active angiogenesis, vascularization, and lymphangiogenesis. Negative feedback regulation is a critical physiological mechanism. Vasohibin-1 (VASH1) was recently discovered as a novel endothelium-derived negative feedback regulator of vascularization. We therefore investigated the expression of VASH1 in the bovine CL. Expression of VASH1 mRNA and protein was predominantly localized to luteal endothelial cells (LECs). VASH1 expression in the CL was constant through the early to late luteal phases and decreased during CL regression relating with the action of luteolytic prostaglandin $F_{2\alpha}$ *in vivo*. To investigate the role of VASH1, we determined whether VASH1 treatment affects angiogenesis and/or lymphangiogenesis using LECs and lymphatic endothelial cells (LyECs) *in vitro*. Vascular endothelial growth factor A (VEGFA) stimulated the expression of VASH1 in LECs but not in LyECs, and VASH1 completely blocked VEGFA-induced formation of capillary-like tube structures of LECs and LyECs *in vitro*. In summary, VASH1 is predominantly located on LECs in the bovine CL and inhibits the angiogenic and lymphangiogenic actions of VEGFA. Bovine CL therefore has a VEGFA–VASH1 system that may be involved in regulation of luteal function, especially in the development of the CL. The results indicate that VASH1 has the potential to act as a negative feedback regulator of angiogenesis and lymphangiogenesis in the CL in cows.

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Introduction

The corpus luteum (CL) is an essential organ for maintaining pregnancy and functions by secreting progesterone (P_4 ; Rodgers *et al.* 1988). The bovine CL develops rapidly within 2–3 days after ovulation; this development is accompanied by active angiogenesis, vascularization, and lymphangiogenesis (Augustin *et al.* 1995, Schams & Berisha 2004, Nitta *et al.* 2011). The CL is a highly vascularized organ with a vascular density exceeding those of most tumors. If pregnancy is not successfully established, the CL is functional for only 17–18 days and must then regress within a few days to allow for the next cycle of ovulation (McCracken *et al.* 1981). In the cow, luteal regression is induced by uterine prostaglandin (PG) $F_{2\alpha}$ together with disruption of the vascular system, including blood and lymphatic vessels (Niswender *et al.* 1976, Acosta *et al.* 2002, Hojo *et al.* 2009, Nitta *et al.* 2011). Vascular systems and endothelial cells within the CL thus have essential roles in luteal function.

Angiogenesis, the formation of new blood vessels, is crucial for various developmental or remodeling processes involved in physiological and pathophysiological

conditions. Physiological angiogenesis occurs not only during organ growth and development and wound healing but also during reproduction in the uterus and ovary. Angiogenesis is generally regulated by the local balance between stimulatory and inhibitory angiogenic factors (Sato & Sonoda 2007, Sato 2011). The most important stimulatory angiogenic factors are vascular endothelial growth factor A (VEGFA) and basic fibroblast growth factor (FGF2), while, angiogenesis inhibitors include thrombospondin-1 (Good *et al.* 1990), pigmented epithelium-derived factor (Tombran-Tink *et al.* 1991), angiostatin (O'Reilly *et al.* 1994), and endostatin (O'Reilly *et al.* 1997). Negative feedback regulation is a critical physiological mechanism and has been demonstrated to control a wide range of phenomena. However, little is known about endogenous inhibitors of angiogenesis in the bovine CL.

Watanabe *et al.* (2004) recently discovered a novel endothelium-derived negative feedback regulator of angiogenesis, vasohibin-1 (VASH1), by searching for VEGFA-inducible genes in human endothelial cells using DNA microarray analysis and isolating the previously uncharacterized gene *VASH1*. Human

VASH1 protein is composed of 365 amino acid residues, with no detectable glycosylation. VASH1 is predominantly expressed on endothelial cells based on the immunohistochemistry data in humans and mice. Recombinant VASH1 inhibits endothelial cell migration, proliferation, and network formation *in vitro* as well as angiogenesis *in vivo* (Watanabe *et al.* 2004, Hosaka *et al.* 2009, Heishi *et al.* 2010). Furthermore, the coadministration of VASH1 with VEGFA has been reported to block, almost completely, not only VEGFA-induced angiogenesis but also lymphangiogenesis in the mouse cornea (Heishi *et al.* 2010).

Given its role in angiogenesis and lymphangiogenesis, we hypothesized that VASH1 is involved in the function of the CL, but expression of VASH1 in the bovine CL has not been described. Therefore, in the present study, we investigated the local production and possible role of VASH1 in the bovine CL, by examining the mRNA expression and localization of VASH1 during the formation, maturation, and regression of the CL in cows. We also explored whether VASH1 is involved in angiogenesis and/or lymphangiogenesis using luteal endothelial cells (LECs) and lymphatic endothelial cells (LyECs) *in vitro*.

Results

VASH1 mRNA and protein expression in the CL during the estrous cycle and PGF_{2α}-induced luteolysis

Figure 1A, B, C and D shows VASH1 mRNA and protein expression in the CL during the estrous cycle (Fig. 1A and B) and PGF_{2α}-induced luteolysis (Fig. 1C and D). During the estrous cycle, VASH1 mRNA expression significantly increased from the early to the mid-luteal CL (Fig. 1A, $P < 0.05$), while VASH1 protein expression remained constant from the early through the late luteal phase (Fig. 1B). In the regressing CL, expression of VASH1 mRNA and protein was significantly decreased (Fig. 1A and B, $P < 0.05$). During PGF_{2α}-induced luteolysis, expression levels of VASH1 mRNA and protein in the CL significantly decreased beginning at 30 min or 2 h respectively (Fig. 1C and D, $P < 0.05$). After PGF_{2α} injection, plasma P₄ (0 h = 5.3 ± 0.5 ng/ml) significantly decreased at 30 min and 2 h (about 70% of 0 h) and was further reduced at 12 h by 25% of 0 h (data not shown).

Localization of VASH1 mRNA and protein in the CL

VASH1 mRNA expression was detected in various cell and tissue types by RT-PCR (Fig. 2A). The bovine mid-cycle CL expressed VASH1 mRNA. Cultured (passages 5–8) LECs also showed relatively high expression of VASH1 mRNA, while cultured (passages 5–8) iliac LyECs and steroidogenic luteal cells (SCs) expressed VASH1 mRNA at lower levels. These results suggest that the low detectable signal of VASH1 mRNA in isolated LyECs and

Figure 1 Changes in VASH1 mRNA and protein expression in the bovine CL. VASH1 mRNA expression (A) and protein expression (B) in the bovine CL during the estrous cycle ($n = 4$ –5/stage). VASH1 mRNA (C) and protein (D) expression in the bovine CL during PGF_{2α}-induced luteolysis ($n = 5$ /stage). All values are shown as mean $\pm$ S.E.M. Different superscript letters (a and b) and * (compared with 0 h) indicate significant differences ($P < 0.05$) as determined by ANOVA followed by Bonferroni's multiple comparison test.

SCs could be due to contamination of the cell preparations with microvascular cells.

To determine the localization of VASH1 protein depending on cell types, VASH1 immunohistochemical staining was compared with that against von Willebrand factor (VWF, a marker of the vascular endothelial cells) or LYVE1 (a marker of LyECs). Figure 2B, C, D and E shows a series of sections of the bovine mid-cycle CL stained for VWF–VASH1 and LYVE1–VASH1 respectively. Figure 2B shows VWF-positive endothelial cells in the large blood vessels (black arrowhead) and microcapillary vessels (red arrowheads), while Fig. 2C shows that VASH1 protein was localized to the same microcapillary vessels as VWF (red arrowheads) but not to the large blood vessels (white arrowhead). Similarly, Fig. 2D shows LYVE1-positive LyECs in the bovine CL (green arrowheads), but Fig. 2E shows that the VASH1 protein did not co-localize with LYVE1 (gray arrowheads). Like VASH1, LYVE1 was not detected in VWF-positive large blood vessels (Fig. 2D, white arrowhead). In the present study, VASH1 protein was barely detectable in SCs, suggesting that the LECs of microcapillary and/or small blood vessels are the predominant VASH1-expressing cells in the bovine CL.

Effect of VEGFA and VASH1 on VEGFA and VASH1 expression in LECs and LyECs

We investigated the effects of VEGFA and VASH1 on LECs and LyECs. Figure 3 shows the changes in VEGFA and VASH1 mRNA and protein expression levels in LECs and LyECs after treatment with VEGFA or VASH1 (1, 10 or 100 ng/ml). VEGFA at 10 ng/ml significantly increased

Figure 2 mRNA expression and localization of *VASH1* in the bovine CL. Panel A shows the mRNA expression of *VASH1* in various cell or tissue types, such as cultured LECs, cultured iliac LyECs, SCs, and CL (mid-luteal phase). M indicates DNA mass ladder. Panels B, C, D and E show the immunohistochemical localization of *VASH1* in the mid-CL during the estrous cycle. VWF-positive cells were detected among the endothelial cells of both large blood and microcapillary vessels (B). The vascular endothelial cells in microcapillary vessels (C, red arrowheads) but not large blood vessels (white arrowhead) also stained for *VASH1*. D shows LYVE1-positive lymphatic endothelial cells in the bovine CL (green arrowheads), while (E) shows that *VASH1* protein did not co-localize with LYVE1 (gray arrowheads). The specificity of the staining was monitored in a negative control section (F) by replacing the antibody with goat anti-rabbit IgG. The scale bars in all images represent 50 μ m.

VASH1 mRNA and protein expression in LECs (Fig. 3A and B), while *VASH1* at 1–100 ng/ml had no effect on *VEGFA* mRNA expression in LECs (Fig. 3C). In LyECs, neither *VEGFA* nor *VASH1* treatment had any effect on *VASH1* or *VEGFA* mRNA expression (Fig. 3D and E).

Effects of *VEGFA* and *VASH1* on proliferation of LECs and LyECs

We next examined the effects of *VEGFA* and *VASH1* on the proliferation of LECs and LyECs *in vitro* (Fig. 4A and B). The data show that neither *VEGFA* (1–100 ng/ml), nor *VASH1* (100 ng/ml) nor a combination of *VEGFA* and *VASH1* had any effect on the proliferation of LECs (Fig. 4A). In LyECs, *VEGFA* (10 and 100 ng/ml) significantly stimulated

cell proliferation for 24 h in culture. Although *VASH1* had no individual effect on the proliferation of LyECs, it did significantly inhibit *VEGFA*-induced cell proliferation (Fig. 4B).

Effects of *VEGFA* and *VASH1* on capillary-like tube formation by LECs and LyECs

We next examined the effects of *VEGFA* and *VASH1* on capillary-like tube formation of LECs and LyECs *in vitro* using a matrigel assay (Figs 5 and 6). The data show that *VEGFA* at 10 and 100 ng/ml significantly stimulated the formation of capillary-like tube structures (a linear array of cells with junctional complexes, not a cylindrical structure) by both LECs and LyECs, compared with that by control cells, 8 h posttreatment (Figs 5A and 6A). In both LECs and LyECs, while *VASH1* had no individual effect on capillary-like tube formation (Figs 5B, G and 6B and G), it significantly inhibited the formation of *VEGFA*-induced capillary-like tube structures (Figs 5B, C and F and 6B, C and F). Typical images of tube formation by control, *VEGFA*-treated, *VASH1*-treated, and *VEGFA* and *VASH1* combination-treated LECs and LyECs are shown in Figs 5B, C, D and G and 6B, C, D and G respectively.

Discussion

In the present study, we have evaluated the expression of *VASH1* in bovine CL tissue during the estrous cycle and $\text{PGF}_{2\alpha}$ -induced luteolysis. Both immunohistochemical and mRNA expression analyses showed that *VASH1* was predominantly localized to LECs relative to SCs and LyECs. *VASH1* expression was consistent from the early through the late luteal phases but decreased in the CL during regression due to the luteolytic action of $\text{PGF}_{2\alpha}$. *VEGFA* treatment stimulated the expression of *VASH1* in LECs, while *VASH1* treatment completely blocked *VEGFA*-induced capillary-like tube structure formation by LECs and LyECs *in vitro*. Therefore, the bovine CL has a *VEGFA*–*VASH1* system that may be involved in luteal function, especially the development of the CL in cows.

Watanabe *et al.* (2004) proposed that *VASH1* is an endothelium-derived negative feedback regulator of angiogenesis. *VASH1* has been reported to be expressed predominantly in the endothelial cells of the artery, corneal, retina, lymph node, endometrium, glomerulus, and tumors in humans (Yamashita *et al.* 2006, Yoshinaga *et al.* 2008, Hosaka *et al.* 2009, Tamaki *et al.* 2009) and mice (Kimura *et al.* 2009, Heishi *et al.* 2010, Li *et al.* 2010). Expression studies in human endothelial cells revealed that *VASH1* protein localized to the nucleus and cytoplasm (Watanabe *et al.* 2004, Kern *et al.* 2008). In cows in the present study, strong expression of both *VASH1* mRNA and protein was detected in LECs. Moreover, SCs in the CL and LyECs in cows did express very low levels of *VASH1*. This finding is consistent with previous studies in which

Figure 3 VASH1 and VEGFA expression profiles in LECs and LyECs. This figure shows the effects of VEGFA or VASH1 on the expression of VASH1 or VEGFA in cultured LECs (A, B and C) and cultured iliac LyECs (D and E) respectively. VEGFA stimulated VASH1 mRNA expression in cultured LECs (A) but not LyECs (D). VEGFA also stimulated VASH1 protein expression in cultured LECs (B). Representative photographs of western blots for VASH1 (42 kDa) and β -actin (37 kDa) are shown (B). In contrast, VASH1 had no effect on VEGFA mRNA expression in either cultured LECs (C) or cultured iliac LyECs (E). All values are shown as mean $\pm$ S.E.M. ($n=4$ /group). *Significant difference ($P<0.05$, compared with control) as determined by ANOVA followed by Bonferroni's multiple comparison test.

weak VASH1 expression was observed in aortic smooth muscle cells and fibroblast, peripheral blood mononuclear cells, and synovial lining cells in humans (Kern *et al.* 2008, Miyake *et al.* 2009). In addition, VASH1 expression was observed in blood, vascular tissue, spleen, heart, placenta, embryo, and brain in humans, mice, and chickens (Shibuya *et al.* 2006, Nimmagadda *et al.* 2007), suggesting that VASH1 might have further functions not only in angiogenesis but also in organ development or immunobiology.

VASH1 was discovered as a VEGFA-induced anti-angiogenic factor (Watanabe *et al.* 2004). Recombinant VASH1 completely inhibited network formation by endothelial cells *in vitro* as well as angiogenesis *in vivo* (Watanabe *et al.* 2004). Additionally, administration of recombinant or adenovirus vector encoded

VASH1 blocked not only VEGFA-induced angiogenesis but also lymphangiogenesis in the mouse cornea (Heishi *et al.* 2010). These findings indicated that VASH1 is a key negative regulatory component of the vascular system. Therefore, in the present study, we investigated the expression profile of VASH1 and its function in LECs and LyECs *in vitro*. It has been clearly demonstrated that VEGFA stimulates the expression of both VASH1 mRNA and protein in a dose- and time-dependent manner in human endothelial cells *in vitro* (Watanabe *et al.* 2004). As in previous studies, VEGFA in the present study increased both VASH1 mRNA and protein expression in the bovine LECs but not in LyECs. Importantly, VASH1 treatment completely suppressed VEGFA-stimulated capillary tube-like structure formation by both LECs and LyECs and proliferation of LyECs. These findings suggest that VASH1 is a key negative feedback regulatory factor of vascularization, including angiogenesis and lymphangiogenesis.

The bovine CL is a highly vascularized organ and produces various angiogenic factors. The highest expression levels of VEGFA and FGF2 were detected

Figure 4 VEGFA and VASH1-induced cell proliferation of LECs and LyECs. The effects of VEGFA and VASH1 on the proliferation of cultured LECs (A) and cultured iliac LyECs (B) are shown. VEGFA (1–100 ng/ml), VASH1 (100 ng/ml), and a combination of VEGFA and VASH1 had no effect on proliferation of cultured LECs (A). In cultured iliac LyECs, VEGFA (10 and 100 ng/ml) significantly stimulated cell proliferation. Although VASH1 had no individual effect on the proliferation of cultured iliac LyECs, it significantly inhibited VEGFA-induced cell proliferation (B). All values are shown as mean $\pm$ S.E.M. ($n=4$ /group). Different superscript letters (a and b) indicate significant differences ($P<0.05$) as determined by ANOVA followed by Bonferroni's multiple comparison test.

Figure 5 VEGFA and VASH1-induced capillary-like tube formation by LECs. The effects of VEGFA and VASH1 on capillary-like tube formation by cultured LECs *in vitro* in a matrigel assay are shown. VEGFA at 10 and 100 ng/ml significantly stimulated the capillary-like tube structure formation by cultured LECs relative to that by control cells at 8 h posttreatment (A). VASH1 had no individual effect on capillary-like tube formation, but it significantly inhibited VEGFA-induced capillary-like tube structure formation. Typical images of tube formed by cultured LECs in each experimental group are shown in B, C, D, E, F and G. All values are shown as mean $\pm$ S.E.M. ($n=4$ /group). Different superscript letters (a and b) indicate significant differences ($P<0.05$) as determined by ANOVA followed by Bonferroni's multiple comparison test.

during development of the CL and were continuously expressed in the mature CL in cows (Schams *et al.* 1994, Berisha *et al.* 2000). VASH1 protein expression was also at its highest level in the early CL, whereas VASH1 mRNA expression was increased from the early to mid-CL in the present study. Excessive production of angiogenic factors such as VEGFA and FGF2 may induce disordered angiogenesis and lymphangiogenesis, suggesting that VASH1 is involved in the homeostatic regulation of the luteal environment to prevent inappropriate vascular system development. In fact, VASH1 had no individual effect on angiogenesis but did suppress angiogenesis and lymphangiogenesis induced by VEGFA or FGF2 *in vivo* and *in vitro* in both the present study and previous reports (Watanabe *et al.* 2004, Shibuya

et al. 2006, Heishi *et al.* 2010). Additionally, VEGFA and FGF2 are expressed at higher levels in breast carcinoma and endometrioid adenocarcinoma in humans (Yoshinaga *et al.* 2008). In the mouse tumor model induced by the injection of Lewis lung carcinoma cells into the subcutaneous tissue, tumors grew bigger in VASH1^{-/-} mice than in wild-type mice, and the vascular area was increased and the tumor vessels were more immature, lacking mural cells in VASH1^{-/-} mice (Hosaka *et al.* 2009). These data indicate that endogenous VASH1 participates in the inhibition of tumor angiogenesis and the maturation of vascular vessels.

In the present study, VASH1 expression significantly decreased in the regressing CL whether the regression was due to the estrous cycle or PGF_{2 α} -induced luteolysis. This

Figure 6 VEGFA and VASH1-induced capillary-like tube formation by LyECs. The effects of VEGFA and VASH1 on capillary-like tube formation of cultured iliac LyECs *in vitro* in a matrigel assay are shown in figure. VEGFA at 10 and 100 ng/ml significantly stimulated capillary-like tube structure in cultured iliac LyECs relative to that by control cells at 8 h posttreatment (A). VASH1 had no individual effect on capillary-like tube formation, but it significantly inhibited VEGFA-induced capillary-like tube structure. Typical images of tube formed by cultured iliac LyECs in each experimental group are shown in B, C, D, E, F and G. All values are shown as mean $\pm$ S.E.M. ($n=4$ /group). Different superscript letters (a and b) indicate significant differences ($P<0.05$) as determined by ANOVA followed by Bonferroni's multiple comparison test.

finding is supported by previous studies in which the expression of VEGFA was acutely inhibited and redundant vascular system gradually disrupted during luteolysis in cows (Neuvians *et al.* 2004, Hojo *et al.* 2009, Berisha *et al.* 2010). On the other hand, it is well known that acceleration of luteolysis mobilizes macrophages into the luteal tissue via monocyte chemoattractant protein-1 (MCP1) expression (Penny 2000, Cavicchio *et al.* 2002, Townson *et al.* 2002). Interestingly, VASH1 administration significantly suppressed macrophage infiltration and the expression of MCP1 as well as inhibiting angiogenesis in diabetic mice (Nasu *et al.* 2009). The infiltration of macrophages was also significantly reduced by adenovirus-mediated VASH1 injection into the mouse femoral artery (Yamashita *et al.* 2006). Moreover, macrophage numbers and MCP1 expression are at low levels while VASH1 expression is at higher levels in the mid to late luteal phases compared with the regressing CL (Penny 2000, Townson *et al.* 2002). Therefore, it has been suggested that downregulation of VASH1 during luteolysis contributes to a suitable microenvironment for recruiting macrophages into the CL. However, Yamashita *et al.* (2006) suggested that the inhibition of macrophage infiltration may be due to the inhibition of angiogenesis, as VASH1 did not affect the migration of macrophages in that study. Further investigation is required to determine 1) why VASH1 is downregulated during luteolysis, 2) the relationships between the decrease of VASH1, and the potential role of $\text{PGF}_{2\alpha}$, $\text{TNF}\alpha$, and $\text{IFN}\gamma$, and 3) whether VASH1 regulates the migration of immune cells directly or indirectly in cows.

Antibody against VEGFR2 but not FLT1 (VEGFR1) completely blocked the intracellular signaling cascade that mediates induction of VASH1 by VEGFA (Shimizu *et al.* 2005). Protein kinase C (PRKC (PKC)) is an important downstream intracellular signaling mediator of VEGFR2. Upon VEGFA stimulation, the activation of PKC δ peaked at 10 min, followed by increased VASH1 expression, and a specific inhibitor of PKC δ completely suppressed the upregulation of VASH1 induced by VEGFA (Shimizu *et al.* 2005). These findings demonstrated that the induction of VASH1 expression is initiated by VEGFR2 signaling and PKC δ is thought to play a principal role in VASH1 expression. As negative regulator of vascularization, VASH1 should act to inhibit the angiogenic and lymphangiogenic activity of VEGFA. However, the mechanisms by which VASH1 acts are controversial; treatment with adenovirus encoded-VASH1 suppressed the increase of VEGFR2 expression and its phosphorylation in mice (Nasu *et al.* 2009, Zhou *et al.* 2010), whereas VASH1 did not inhibit VEGFA-induced VEGFR2 phosphorylation in human umbilical vein endothelial cells (Watanabe *et al.* 2004). In the present study, VASH1 treatment did not affect the mRNA expression of *VEGFR2* in either LECs or LyECs in cows (data not shown). In addition, the specific VASH1 receptor is yet to be discovered. A correct understanding of the VEGFA–VASH1 system,

including its functions, signaling, and receptor will guide novel research into both physiological and pathophysiological vascularization.

In summary, the present results demonstrate the expression of VASH1, especially in blood vascular endothelial cells in the bovine CL and clearly show that VASH1 inhibits VEGFA-stimulated capillary like-tube formation by both LECs and LyECs *in vitro*. Taken together, these results indicate that VASH1 may act as a negative feedback regulator of angiogenesis and lymphangiogenesis in the CL in cows.

Materials and Methods

The CLs were collected at the Field Center of Animal Science and Agriculture, Obihiro University and the Clinic for Cattle, University of Veterinary Medicine Hannover, Germany. Each experimental procedure complied with the ethics committee on animal right 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, 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 was purchased from Invitrogen Corporation; cell culture plates (24, 48, or 96 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 bovine VEGFA was purchased from Kingfisher Biotech, Inc. (St Paul, MN, USA); Protease Inhibitor Cocktail and WST-1 were purchased from Roche; 70 μm filter (Cell Strainer, REF 352350) was purchased from BD Falcon (Franklin Lakes, NJ, USA); PVDF membranes were purchased from Bio-Rad Laboratories (Hercules, CA, USA); lymphoprep was purchased from Axis-Shield (Oslo, Norway); HRP-conjugated anti-rabbit IgG antibodies were purchased from GE Healthcare (Ltd, Buckinghamshire, UK); HRP-conjugated anti-mouse IgG antibodies were purchased from Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA); anti-human VWF rabbit polyclonal antibody (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); anti-human-VASH1 rabbit polyclonal antibody was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, UK); VASH1 recombinant protein was purchased from Abnova Corporation (Taipei, Taiwan).

Collection of the bovine CL

CLs during the estrous cycle

To investigate the localization of VASH1, ovaries were collected from a local slaughterhouse in Obihiro, Japan. The stage of the estrous cycle was defined by macroscopic observation of the ovaries (follicles and CL; size, surface, and color) and the uterus (size, consistency, connective tissue, and mucus) as described previously (Miyamoto & Schams 1991, Miyamoto *et al.* 2000). The stages of the estrous cycle ($n=4-5$ in each stage) were estimated as follows: early (days 3–5), mid (days 8–12), late (days 13–15), and regression (days 18 <).

CLs during PGF_{2α}-induced luteolysis

For collecting the CL during luteolysis, 20 normal cyclic German Holstein cows in University of Veterinary Medicine Hannover, Germany were used. The day of estrus was designated as day 0. Cows ($n=5$ for each time point) at the mid-luteal phase (days 10–12) were injected with PGF_{2α} in intramuscular (0 h) (25 mg Dinoprost=PGF_{2α}, 5.0 ml Dinolytic, Pfizer, Berlin, Germany), and ovaries were collected by ovariectomy (Shirasuna *et al.* 2010) through the vagina at 0 (before PGF_{2α} injection), 30 min, 2 h (as the phase of functional luteolysis), and 12 h (as the phase of starting structural luteolysis).

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 µl TRIzol reagent and stored at -80°C until analysis. Immunohistochemical and molecular analyses were performed in Obihiro University, Japan.

Culture of LECs, LyECs, and luteal cells

LEC culture

LECs were used in this study following the method described by Spanel-Borowski (1991). Cytokeratin-negative LECs isolated from the CLs of the cows during the mid-luteal phase were used as described previously (Spanel-Borowski 1991, Tschedschilsuren *et al.* 2002). Briefly, small pieces of the CL tissue were disintegrated and the fragments were serially sieved through nylon meshes with pore sizes of 70 µm. Cells were seeded on 24-well culture plate, and colony of endothelial cells was monitored and selected by microscopy observation (Spanel-Borowski 1991, Tschedschilsuren *et al.* 2002). These cells maintained a stable phenotype during long-term culture. LECs were grown on plates precoated with 1% Vitrogen in DMEM/F-12 medium containing 5% FBS, 2.2% NaHCO₃, 0.1% gentamicin solution, and 1% amphotericin B solution.

All experiments in the present study were carried out on LECs from passages 5 to 8. To determine the purity of isolated LECs, these LECs expressed typical endothelial cell marker, VWF, by immunohistochemical analysis (data not shown).

LyEC culture

LyECs were isolated in our laboratory using the method described by Nitta *et al.* (2011). Briefly, fresh sections of the internal iliac lymphatic vessels were taken from cattle, and the lymph 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 LyECs were grown on plates precoated with 1% Vitrogen in DMEM/F-12 medium containing 5% FBS, 2.2% NaHCO₃, 0.1% gentamicin solution, and 1% amphotericin B solution. All experiments in the present study were carried out on LyECs from passages 5 to 8. To determine the purity of isolated LyECs, these LyECs expressed typical LyEC marker, LYVE1, by immunohistochemical analysis (data not shown) (Nitta *et al.* 2011).

Steroidogenic luteal cell (SC) culture

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 10–12) by macroscopic observation of the ovary as described previously (Miyamoto *et al.* 1992). SCs were used in this study following the method described by Klipper *et al.* (2004). Briefly, SCs were isolated from the bovine mid-CL (days 8–12 of the estrous cycle) using magnetic tosylactivated beads coating with BS-1 lectin (binds glycoproteins on the bovine endothelial cells), indicating that BS-1-positive cells are endothelial cells. In the present study, BS-1-negative cells were assessed as SCs as described previously (Klipper *et al.* 2004). SCs were grown in DMEM/F-12 medium containing 5% FBS, 2.2% NaHCO₃, 0.1% gentamicin solution, and 1% amphotericin B solution. To determine the purity of isolated SCs, these SCs predominantly expressed typical steroidogenic luteal cell marker, steroid acute regulatory protein by real-time RT-PCR analysis (data not shown). All cells including LECs, LyECs, and SCs were isolated in Obihiro University, Japan.

Before treatment of VEGFA and VASH1, LECs and LyECs were washed twice with PBS. Cells were incubated in DMEM/F-12 medium containing 0.1% FBS, 2.2% NaHCO₃, 0.1% gentamicin solution, and 1% amphotericin B solution for 24 h at 37°C with the following additions: control (no further addition), VEGFA (1, 10, or 100 ng/ml), or VASH1 (1, 10, or 100 ng/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 and protein expression. At least four experiments were performed, with each concentration of agents tested with two replicates/experiments.

Immunohistochemistry

Serial sections, 7 µm thick, of the CLs were mounted on glass microscope slides coated with APS and immunohistochemistry

for VASH1, 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% H₂O₂ 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 VASH1 (diluted 1:100), 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% H₂O₂ followed by nuclear staining with hematoxylin.

Proliferation of LECs and LyECs

LECs and LyECs (5×10^3 cells/well in 96-well plates) were seeded on plates precoated with 1% Vitrogen in DMEM/F-12 medium containing 0.1% FBS, 2.2% NaHCO₃, 0.1% gentamicin solution, and 1% amphotericin B solution, then the cells were exposed to VEGFA (1, 10, or 100 ng/ml), VASH1 (100 ng/ml), or their combinations for 24 h at 37 °C. After 24 h, the cell proliferation was determined by the Cell Proliferation Reagent WST-1 kit (Roche). After adding WST-1 reagent, the cells were incubated for 4 h at 37 °C. The absorbance was read at 450 nm using microplate reader. At least four experiments were performed, with each concentration of agents tested with three replicates/experiments.

Capillary tube formation on matrigel of LECs and LyECs

LEC and LyEC capillary tube formation was evaluated as previously described (Jientaweeboon *et al.* 2011, Nitta *et al.* 2011). 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. The cells (2×10^4 cells/well) were plated in a final volume of 0.5 ml/well of culture medium containing VEGFA (10 or 100 ng/ml), VASH1 (100 ng/ml), or their combinations. 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 (1×1 mm) was analyzed. Data are shown as mean $\pm$ S.E.M. At least four experiments were performed, with each concentration of agents tested with two replicates/experiments.

RNA extraction, cDNA synthesis, and reverse-transcription 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 previously described (Watanabe *et al.* 2006). The mRNA expression levels of VEGFA, VASH1, and β -actin were quantified by RT quantitative PCR (RT-qPCR) as previously described (Watanabe *et al.* 2006). The primers used for real-time PCR were as follows: 5'-attttcaagccgtcctgtgt-3', forward and 5'-tatgtgctggcttggtag-3', reverse for VEGFA (accession no. MN_174216); 5'-cagtaaggattggggatg-3', forward and 5'-accccggttaacaagaagg-3', reverse for VASH1 (accession no. MN_001206803.1), and 5'-ccaaggccaaccgtgagaaaat-3', forward and 5'-ccacattccgtgaggatcttca-3', reverse for β -actin (accession no. MN_173979.3). RT-qPCRs were performed in duplicate in a final volume of 10 µl containing 5 µl of SYBER Green, 2.8 µl of H₂O, 0.1 µl of 50 µM forward and reverse primers, and 2 µl of cDNA template or water (as nontemplate negative control). RT-qPCR conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s, and 72 °C for 20 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 as an internal standard.

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-human-VASH1 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 Bonferroni'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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References

- Acosta TJ, Yoshizawa N, Ohtani M & Miyamoto A 2002 Local changes in blood flow within the early and midcycle corpus luteum after prostaglandin F_{2α} injection in the cow. *Biology of Reproduction* **66** 651–658. (doi:10.1095/biolreprod66.3.651)
- Augustin HG, Braun K, Telemenakis I, Modlich U & Kuhn W 1995 Ovarian angiogenesis. Phenotypic characterization of endothelial cells in a physiological model of blood vessel growth and regression. *American Journal of Pathology* **147** 339–351.
- Berisha B, Schams D, Kosmann M, Amselgruber W & Einspanier R 2000 Expression and tissue concentration of vascular endothelial growth factor, its receptors, and localization in the bovine corpus luteum during estrous cycle and pregnancy. *Biology of Reproduction* **63** 1106–1114. (doi:10.1095/biolreprod63.4.1106)
- Berisha B, Meyer HH & Schams D 2010 Effect of prostaglandin F_{2α} on local luteotropic and angiogenic factors during induced functional luteolysis in the bovine corpus luteum. *Biology of Reproduction* **82** 940–947. (doi:10.1095/biolreprod.109.076752)
- Cavicchio VA, Pru JK, Davis BS, Davis JS, Rueda BR & Townson DH 2002 Secretion of monocyte chemoattractant protein-1 by endothelial cells of the bovine corpus luteum: regulation by cytokines but not prostaglandin F_{2α}. *Endocrinology* **143** 3582–3589. (doi:10.1210/en.2002-220388)
- Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159. (doi:10.1016/0003-2697(87)90021-2)
- Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA & Bouck NP 1990 A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *PNAS* **87** 6624–6628. (doi:10.1073/pnas.87.17.6624)
- Heishi T, Hosaka T, Suzuki Y, Miyashita H, Oike Y, Takahashi T, Nakamura T, Arioka S, Mitsuda Y, Takakura T *et al.* 2010 Endogenous angiogenesis inhibitor vasohibin1 exhibits broad-spectrum antilymphangiogenic activity and suppresses lymph node metastasis. *American Journal of Pathology* **176** 1950–1958. (doi:10.2353/ajpath.2010.090829)
- Hojó T, Al-Zi'abi MO, Skarzynski DJ, Acosta TJ & Okuda K 2009 Changes in the vasculature of bovine corpus luteum during the estrous cycle and prostaglandin F_{2α}-induced luteolysis. *Journal of Reproduction and Development* **55** 512–517. (doi:10.1262/jrd.20257)
- Hosaka T, Kimura H, Heishi T, Suzuki Y, Miyashita H, Ohta H, Sonoda H, Moriya T, Suzuki S, Kondo T *et al.* 2009 Vasohibin-1 expression in endothelium of tumor blood vessels regulates angiogenesis. *American Journal of Pathology* **175** 430–439. (doi:10.2353/ajpath.2009.080788)
- Jiementaveeboon S, Shirasuna K, Nitta A, Kobayashi A, Schuberth HJ, Shimizu T & Miyamoto A 2011 Evidence that polymorphonuclear neutrophils infiltrate into the developing corpus luteum and promote angiogenesis with interleukin-8 in the cow. *Reproductive Biology and Endocrinology* **9** 79. (doi:10.1186/1477-7827-9-79)
- Kern J, Bauer M, Rychli K, Wojta J, Ritsch A, Gastl G, Gunsilius E & Untergasser G 2008 Alternative splicing of vasohibin-1 generates an inhibitor of endothelial cell proliferation, migration, and capillary tube formation. *Arteriosclerosis, Thrombosis, and Vascular Biology* **28** 478–484. (doi:10.1161/ATVBAHA.107.160432)
- Kimura H, Miyashita H, Suzuki Y, Kobayashi M, Watanabe K, Sonoda H, Ohta H, Fujiwara T, Shimosegawa T & Sato Y 2009 Distinctive localization and opposed roles of vasohibin-1 and vasohibin-2 in the regulation of angiogenesis. *Blood* **113** 4810–4818. (doi:10.1182/blood-2008-07-170316)
- Klipper E, Gilboa T, Levy N, Kisliouk T, Spanel-Borowski K & Meidan R 2004 Characterization of endothelin-1 and nitric oxide generating systems in corpus luteum-derived endothelial cells. *Reproduction* **128** 463–473. (doi:10.1530/rep.1.00271)
- Li D, Zhou K, Wang S, Shi Z & Yang Z 2010 Recombinant adenovirus encoding vasohibin prevents tumor angiogenesis and inhibits tumor growth. *Cancer Science* **101** 448–452. (doi:10.1111/j.1349-7006.2009.01388.x)
- McCracken JA, Schramm W, Barcikowski B & Wilson L Jr 1981 The identification of prostaglandin F_{2α} as a uterine luteolytic hormone and the hormonal control of its synthesis. *Acta Veterinaria Scandinavica. Supplementum* **77** 71–88.
- Miyake K, Nishida K, Kadota Y, Yamasaki H, Nasu T, Saitou D, Tanabe K, Sonoda H, Sato Y, Maeshima Y *et al.* 2009 Inflammatory cytokine-induced expression of vasohibin-1 by rheumatoid synovial fibroblasts. *Acta Medica Okayama* **63** 349–358.
- Miyamoto A & Schams D 1991 Oxytocin stimulates progesterone release from microdialyzed bovine corpus luteum *in vitro*. *Biology of Reproduction* **44** 1163–1170. (doi:10.1095/biolreprod44.6.1163)
- Miyamoto A, Okuda K, Schweigert FJ & Schams D 1992 Effects of basic fibroblast growth factor, transforming growth factor-β and nerve growth factor on the secretory function of the bovine corpus luteum *in vitro*. *Journal of Endocrinology* **135** 103–114. (doi:10.1677/joe.0.1350103)
- Miyamoto Y, Skarzynski DJ & Okuda K 2000 Is tumor necrosis factor α a trigger for the initiation of endometrial prostaglandin F_{2α} release at luteolysis in cattle? *Biology of Reproduction* **62** 1109–1115. (doi:10.1095/biolreprod62.5.1109)
- Nasu T, Maeshima Y, Kinomura M, Hirokoshi-Kawahara K, Tanabe K, Sugiyama H, Sonoda H, Sato Y & Makino H 2009 Vasohibin-1, a negative feedback regulator of angiogenesis, ameliorates renal alterations in a mouse model of diabetic nephropathy. *Diabetes* **58** 2365–2375. (doi:10.2337/db08-1790)
- Neuvians TP, Berisha B & Schams D 2004 Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) expression during induced luteolysis in the bovine corpus luteum. *Molecular Reproduction and Development* **67** 389–395. (doi:10.1002/mrd.20032)
- Nimmagadda S, Geetha-Loganathan P, Prols F, Scaal M, Christ B & Huang R 2007 Expression pattern of vasohibin during chick development. *Developmental Dynamics* **236** 1358–1362. (doi:10.1002/dvdy.21134)
- Niswender GD, Reimers TJ, Dieckman MA & Nett TM 1976 Blood flow: a mediator of ovarian function. *Biology of Reproduction* **14** 64–81. (doi:10.1095/biolreprod14.1.64)
- Nitta A, Shirasuna K, Haneda S, Matsui M, Shimizu T, Matsuyama S, Kimura K, Bollwein H & Miyamoto A 2011 Possible involvement of IFNτ in lymphangiogenesis in the corpus luteum during the maternal recognition period in the cow. *Reproduction* **142** 879–892. (doi:10.1530/REP-11-0157)
- O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH & Folkman J 1994 Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79** 315–328. (doi:10.1016/0092-8674(94)90200-3)
- O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR & Folkman J 1997 Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* **88** 277–285. (doi:10.1016/S0092-8674(00)81848-6)
- Penny LA 2000 Monocyte chemoattractant protein 1 in luteolysis. *Reviews of Reproduction* **5** 63–66. (doi:10.1530/ror.0.0050063)
- Rodgers RJ, Mitchell MD & Simpson ER 1988 Secretion of progesterone and prostaglandins by cells of bovine corpora lutea from three stages of the luteal phase. *Journal of Endocrinology* **118** 121–126. (doi:10.1677/joe.0.1180121)
- Sato Y 2011 Is vasohibin-1 for more than angiogenesis inhibition? *Journal of Biochemistry* **149** 229–230. (doi:10.1093/jb/mvq152)
- Sato Y & Sonoda H 2007 The vasohibin family: a negative regulatory system of angiogenesis genetically programmed in endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* **27** 37–41. (doi:10.1161/01.ATV.0000252062.48280.61)
- Schams D & Berisha B 2004 Regulation of corpus luteum function in cattle – an overview. *Reproduction in Domestic Animals* **39** 241–251. (doi:10.1111/j.1439-0531.2004.00509.x)
- Schams D, Amselgruber W, Einspanier R, Sinowatz F & Gospodarowicz D 1994 Localization and tissue concentration of basic fibroblast growth factor in the bovine corpus luteum. *Endocrine* **2** 907–912.

- Shibuya T, Watanabe K, Yamashita H, Shimizu K, Miyashita H, Abe M, Moriya T, Ohta H, Sonoda H, Shimosegawa T *et al.* 2006 Isolation and characterization of vasohibin-2 as a homologue of VEGF-inducible endothelium-derived angiogenesis inhibitor vasohibin. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26** 1051–1057. (doi:10.1161/01.ATV.0000216747.66660.26)
- Shimizu K, Watanabe K, Yamashita H, Abe M, Yoshimatsu H, Ohta H, Sonoda H & Sato Y 2005 Gene regulation of a novel angiogenesis inhibitor, vasohibin, in endothelial cells. *Biochemical and Biophysical Research Communications* **327** 700–706. (doi:10.1016/j.bbrc.2004.12.073)
- Shirasuna K, Sasahara K, Matsui M, Shimizu T & Miyamoto A 2010 Prostaglandin $F_{2\alpha}$ differentially affects mRNA expression relating to angiogenesis, vasoactivation and prostaglandins in the early and mid corpus luteum in the cow. *Journal of Reproduction and Development* **56** 428–436. (doi:10.1262/jrd.10-0040)
- Spanel-Borowski K 1991 Diversity of ultrastructure in different phenotypes of cultured microvessel endothelial cells isolated from bovine corpus luteum. *Cell Tissue Research* **266** 37–49. (doi:10.1007/BF00678709)
- Tamaki K, Moriya T, Sato Y, Ishida T, Maruo Y, Yoshinaga K, Ohuchi N & Sasano H 2009 Vasohibin-1 in human breast carcinoma: a potential negative feedback regulator of angiogenesis. *Cancer Science* **100** 88–94. (doi:10.1111/j.1349-7006.2008.01015.x)
- Tombran-Tink J, Chader GC & Johnson LV 1991 PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. *Experimental Eye Research* **53** 411–414. (doi:10.1016/0014-4835(91)90248-D)
- Townson DH, O'Connor CL & Pru JK 2002 Expression of monocyte chemoattractant protein-1 and distribution of immune cell populations in the bovine corpus luteum throughout the estrous cycle. *Biology of Reproduction* **66** 361–366. (doi:10.1095/biolreprod66.2.361)
- Tscheudschilsuren G, Aust G, Nieber K, Schilling N & Spanel-Borowski K 2002 Microvascular endothelial cells differ in basal and hypoxia-regulated expression of angiogenic factors and their receptors. *Microvascular Research* **63** 243–251. (doi:10.1006/mvre.2001.2346)
- Watanabe K, Hasegawa Y, Yamashita H, Shimizu K, Ding Y, Abe M, Ohta H, Imagawa K, Hojo K, Maki H *et al.* 2004 Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. *Journal of Clinical Investigation* **114** 898–907. (doi:10.1172/JCI21152)
- Watanabe S, Shirasuna K, Matsui M, Yamamoto D, Berisha B, Schams D & Miyamoto A 2006 Effect of intraluteal injection of endothelin type A receptor antagonist on PGF $_{2\alpha}$ -induced luteolysis in the cow. *Journal of Reproduction and Development* **52** 551–559. (doi:10.1262/jrd.18018)
- Yamashita H, Abe M, Watanabe K, Shimizu K, Moriya T, Sato A, Satomi S, Ohta H, Sonoda H & Sato Y 2006 Vasohibin prevents arterial neointimal formation through angiogenesis inhibition. *Biochemical and Biophysical Research Communications* **345** 919–925. (doi:10.1016/j.bbrc.2006.04.176)
- Yoshinaga K, Ito K, Moriya T, Nagase S, Takano T, Niikura H, Yaegashi N & Sato Y 2008 Expression of vasohibin as a novel endothelium-derived angiogenesis inhibitor in endometrial cancer. *Cancer Science* **99** 914–919. (doi:10.1111/j.1349-7006.2008.00777.x)
- Zhou SY, Xie ZL, Xiao O, Yang XR, Heng BC & Sato Y 2010 Inhibition of mouse alkali burn induced-corneal neovascularization by recombinant adenovirus encoding human vasohibin-1. *Molecular Vision* **16** 1389–1398.

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