Delta-like ligand 4 regulates vascular endothelial growth factor receptor 2–driven luteal angiogenesis through induction of a tip/stalk phenotype in proliferating endothelial cells

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Objective: To explore whether the Dll4/Notch-1 signaling pathway modulates vascular endothelial growth factor (VEGF)-dependent luteal angiogenesis and related function, by inducing a tip/stalk phenotype in endothelial cells (ECs).
Design: Experimental laboratory animal study.
Setting: University-affiliated infertility center.
Animal(s): Immature female mice.
Intervention(s): The presence of leading tip ECs in growing luteal vessel was identified by immunofluorescent analysis of Dll4 in the ovaries of hormonally stimulated female mice. The effects of Dll4 inhibition on luteal vessels functionality and related corpus luteum function were assessed by administering a Dll4 blocking antibody or placebo to hormonally stimulated female mice.
Main Outcome Measure(s): Alteration of the tip/stalk phenotype was identified by immunofluorescence analysis of luteal vascular density, Dll4, Notch-1, and VEGF receptor 2 expression. Lectin perfusion was used to assay blood vessel functionality, whereas apoptosis and P levels were quantified to determine the effects on luteal function.
Result(s): Expression of Dll4 was restricted to the tip of growing vessels. Inhibition of Dll4 signaling promotes promiscuous Dll4 expression, leading to increased, but paradoxically, nonfunctional vascularization, which was associated with decreased P levels.
Conclusion(s): The Dll4/Notch-1 signaling pathway has a modulatory role in VEGF-dependent luteal angiogenesis and related function through induction of a tip/stalk phenotype. (Fertil Steril® 2013;100:1768–76. ©2013 by American Society for Reproductive Medicine.)
Key Words: Corpus luteum, angiogenesis, Dll4/Notch-1 pathway, vessel functionality

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Corpus luteum (CL) formation entails the rapid proliferation of steroid-producing granulosa cells and theca cells, which invade the antral cavity, accompanied by ultra-rapid angiogenesis (1). Around the time of ovulation, multiple vascular sprouts originate from the pre-existing theca cell layer vasculature and grow in response to vascular endothelial growth factor (VEGF) in a

perpendicular, centripetal fashion like spokes on a wheel (2). At completion the CL has the highest vascular density of all the organs in the body (3), which allows it to fulfill its task: producing and releasing high amounts of P into the uterus, to allow implantation of an embryo and maintenance of pregnancy (4). Impairment of CL angiogenesis leads to the induction of luteolysis, thus demonstrating a clear link between vascular development and ovarian function (5). Elucidating how ovarian microvasculature is regulated under physiological conditions is key to identifying targets that can be manipulated when abnormal ovarian vascularization causes dysregulated ovarian disorders, such as polycystic ovary syndrome (6) or ovarian hyperstimulation syndrome (7, 8).

It is known that CL angiogenesis in murine models can be blocked by interfering with the VEGF/VEGF receptor 2 (VEGFR2) pathway (9–11), indicating its importance in initiating sprouting angiogenesis, and thus also in maintaining the functionality of the CL (i.e., P secretion into the blood stream) (10). Nevertheless, it is highly unlikely that only one pathway is sufficient to tightly regulate all of the processes required for CL neovascularization, including sprouting, vessel growth guidance, and vessel organization and assembly (12). Given that our understanding of CL angiogenesis regulation is still in its infancy, which complementary factors, aside from VEGF, are required to orchestrate the complete angiogenic symphony during CL neovascularization remains to be defined, although knowledge gained in other organ systems such as retina, in which postnatal angiogenesis has been more deeply studied, can help us to make inferences (13). It is of note that, similar to CL, the newborn murine retina is an empty, avascular structure: immediately after birth, the vascular system starts to develop from the optic disc, extending radially over the superficial layer of the retina (14).

Given the predictable directionality and timing of neovessel growth and sprouting already defined in the retina model, it has been especially useful in monitoring/determining the role of the proteins of interest in the patterning of neovessel growth (13). From all the genes involved in angiogenesis interrogated using the mouse retina model, the interaction between the Dll4-specific Notch ligand, which is primarily expressed in endothelial cells (ECs), and its Notch-1 receptor emerged as a major regulator of the fine-tuning required for VEGF-initiated bulk sprouting–angiogenesis (15). This statement is based on the observation that inhibition of both this ligand and its receptor causes hyperproliferation, over-sprouting, and chaotic retinal vessel growth, which led us to the hypothesis that Dll-4/Notch-1 signaling in ECs finely tunes angiogenesis by inducing a tip/stalk phenotype (14).

During retinal neovascularization specialized ECs, resembling axonal growth cones, are located at the tips of growing capillaries. Unlike their neighboring endothelial stalk cells, which proliferate and form the vascular lumen, endothelial tip cells do not undergo mitosis but rather migrate by extending filopodia that guide the outgrowing capillaries in response to gradients of extracellular matrix–bound VEGF. Endothelial Dll4 expression, which is mostly restricted to tip cells, activates Notch-1 on stalk cells, resulting in restriction of new sprout development by limiting Dll4 expression and thus tips cell formation in the stalk (16). In agreement with this hypothesis, the importance of the VEGF/VEGFR2–Dll4/Notch-1 feedback loop in fine-tuning angiogenesis has also been demonstrated in animal tumor models in which the systemic blockade of Dll4 increased vessel density owing to intensified sprouting and small vessel branch interconnecting. Paradoxically this increased angiogenesis was also associated with poor tumor growth, which is very likely because the newly growing vessels were not functional (17).

On the basis of the descriptive similarities in the angiogenic events that occur in these two organs (10, 13) and the reported presence of some components of the Notch family in luteal blood vessels (18), we used a mouse model to study whether the Dll-4/Notch-1 signaling pathway might have a modulatory role in VEGF-dependent luteal angiogenesis by regulating tip and stalk ECs.

MATERIALS AND METHODS

Animals

Immature 21-day-old or sexually mature 6- to 8-week-old CD1 female mice (Charles River Laboratories International) were used in the study. The study was approved by the Institutional Animal Care Committee at the University of Valencia. All procedures were performed following the guidelines for the care and use of mammals from the National Institutes of Health.

Experimental Design of Mouse Treatments

Experiment 1: Identification of the Tip/Stalk Phenotype in CL Formation. Immature female mice (n = 9) were stimulated with 10 IU pregnant mare serum gonadotropin (PMSG) and after 48 h were administered 10 IU hCG to trigger ovulation and CL formation. Animals were killed at 18 h (n = 3), 24 h (n = 3), and 48 h (n = 3) after hCG administration, at which point both ovaries were excised and cryosectioned. The morphologic features of the tip/stalk phenotype were analyzed in one ovary by immunofluorescent staining for platelet EC adhesion molecule (PECAM), VEGFR2, Dll4, and Notch-1 as further described below. The contralateral ovary was used to try to identify filopodia at the leading edge of endothelial growth (i.e., on the tip cells).

Experiment 2: Evaluation of the Effects of Dll4 Inhibition on the Morphologic Features of the Tip/Stalk Phenotype in CL. Immature female mice (n = 12) were stimulated with gonadotropins as described in experiment 1. Six hours after hCG administration, the animals (n = 6) were given a 10-mg/kg IP injection of a nonspecific human IgG or an anti-Dll4 blocking antibody (BAb; Genentech; YW152F) (17); animals were killed 24 hours after hCG injection. This specific blocking antibody is a humanized phage antibody that effectively blocks Notch-1 interaction with Dll4 but not other Notch ligands (17). The tip/stalk features were analyzed in one ovary, as described in experiment 1, and

the number of mature corpora lutea was counted in the contralateral ovary.

Experiment 3: Evaluation of the Effects of Dll4 Inhibition on the Functionality of Luteal Vasculature and CL. Immature female mice (n = 8) were stimulated with gonadotropins and treated with Dll4 BAb (n = 4) or an unspecific IgG (n = 4) as described in experiment 2. Forty-eight hours after hCG administration the mice were narcotized by IP injection of ketamine and xylacine (170–260 mg/kg and 8.6–13 mg/kg, respectively). Each mouse was perfused via the tail vein with 100 μg of fluorescein isothiocyanate conjugate (FITC)-conjugated *Lycopersicon esculentum* lectin (Vector Laboratories) diluted in 100 μL of saline; 15 minutes later the animals were killed and the ovaries excised. Blood samples were collected and the luteal function evaluated by measuring P levels. The presence of structural luteolysis and the functionality of the luteal vasculature (i.e., the perfusion) were assessed in one of each of the excised ovaries.

Specimen Collection

Blood was obtained via cardiac puncture, to measure P levels. Both ovaries were carefully dissected, embedded in optimal cutting temperature compound, flash frozen in liquid nitrogen, and stored at −80°C until they were frozen-sectioned.

Immunofluorescence

Ovaries were cryosectioned into 8-μm sections at 50-μm intervals and mounted on Superfrost Gold glass slides (Thermo Fisher Scientific). Immunofluorescence analysis of vascularization by staining for PECAM, Dll4, VEGFR-2, and Notch-1 was performed as described in detail in Supplemental Methods (available online). To study PECAM coexpression, dual immunofluorescence in combination with Dll4, VEGFR-2, or Notch-1 antibodies was performed. Additionally, thick cryocuts (40–50 μm) were obtained and used to identify filopodia in the leading edge of EC growth (tip cells).

Combined Intravital Lectin Histofluorescence and PECAM Immunofluorescence

Cryostat sections (10–20 μm) of lectin-perfused tissues were cut, fixed for 20 minutes in acetone at −20°C, air dried, and immunostained against PECAM as described above. Images were acquired in the green (lectin staining) and red (PECAM staining) channels using a digital camera (Nikon Digital Camera, Dxm1200F) coupled to a fluorescence microscope (Nikon Eclipse E400). Fluorescein isothiocyanate conjugate (green)/PECAM (red) fluorescent signals inside CL structures were segmented and processed as described in the data analysis section below.

Serum P Levels

Serum P levels were measured using a competitive chemiluminescent immunoassay (Diagnostic Products/Siemens).

Measurement of Apoptosis

Apoptotic cells were detected using an ApopTag ISOL Dual Fluorescence Apoptosis Detection Kit (DNase types I and II; Millipore) using 8-μm sections following previously described methodology (10, 19).

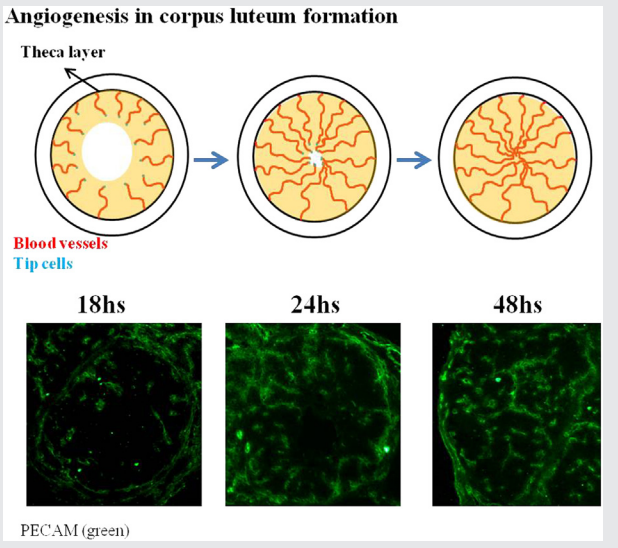
Data Analysis

Quantitative analysis of the area immunostained for PECAM (vascularization), Dll4, Notch-1, and VEGFR2 was performed as previously described (10) and expressed as the percentage of the area of interest per total area in each CL assessed. The percentage of functional blood vessels was determined by using the FITC (lectin-stained) area/PECAM-stained area in each CL multiplied by 100. Apoptosis was estimated following previously described methodology (19) and expressed as the percentage of apoptotic cells per square millimeter. At least 24 individual CL were used for the determination of each of the parameters mentioned above. The total number of mature corpora lutea was determined by visually counting them in the sections from one ovary from each animal by two independent observers and was expressed as the average number of mature corpora lutea per single ovary.

Statistics

Statistical analyses were performed using the Statistical Package for Social Science, version 15.0 (SPSS). Data are

FIGURE 1



Angiogenesis in CL formation. Schematic representation and illustrative images of luteal neoangiogenesis. Images show staining pattern for PECAM (vascularization, *green*) in sections of ovaries from PMSG+hCG-stimulated mice, at different time points of CL development. Note how vascular sprouts in the developing CL are longitudinal structures "arising" perpendicularly from the circular theca layer, similar to spokes on a wheel. Neovessels grow until all the CL is completely filled with blood vessels, approximately 48 hours before hCG. Original magnification, ×100.

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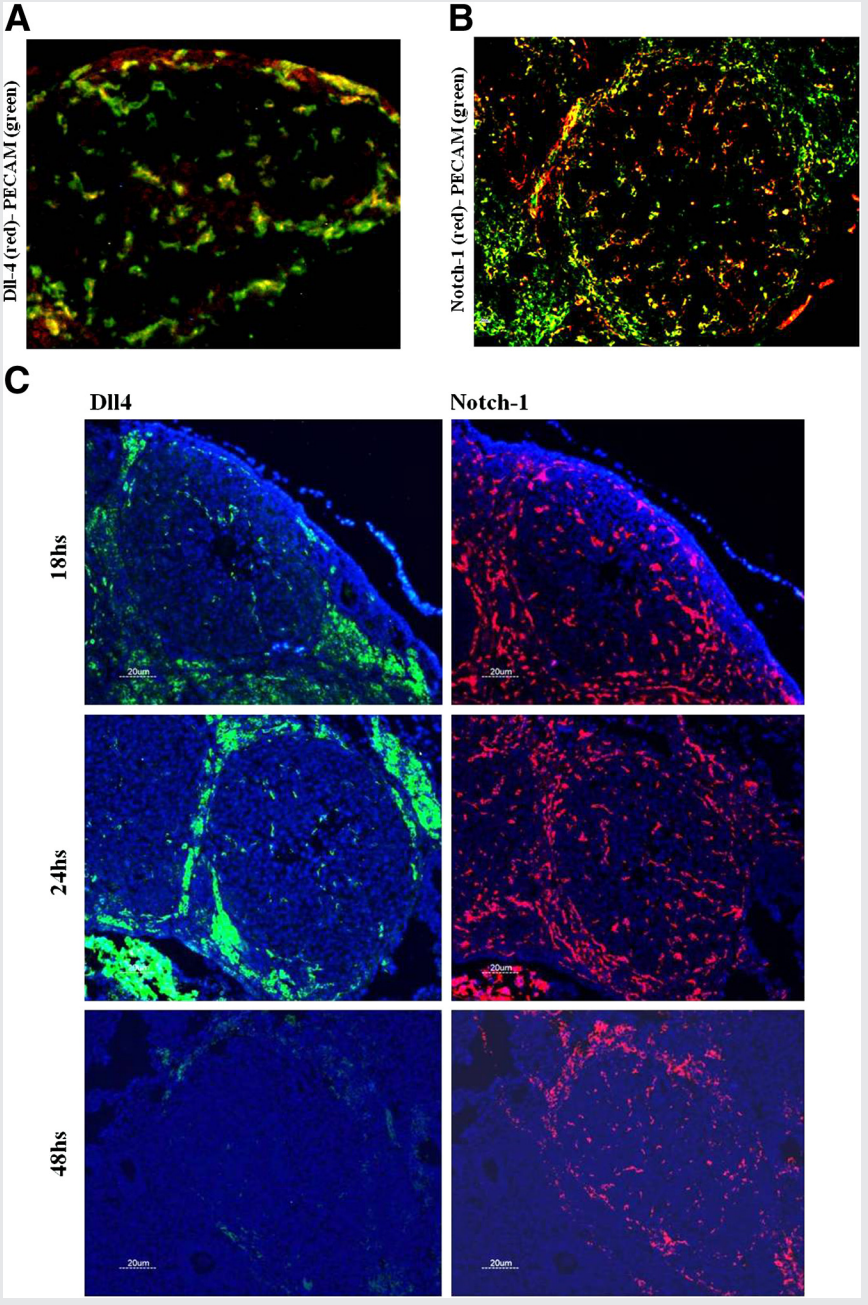
FIGURE 2



Identification of features of the tip/stalk phenotype in developing corpora lutea. Images show double immunofluorescent detection of (A) Dll4 and (B) Notch-1 in combination with PECAM (EC marker) in the ovaries from PMSG+hCG-stimulated mice, 24 hours after hCG administration. Nuclei (*blue*) were stained with 6-diamino-2-phenylindole. Note that most of Dll4 and Notch-1 expression coincides with luteal vasculature (areas in *yellow* represent overlapping). (C) Immunofluorescent detection of Notch-1 (*red*), Dll4 (*green*), in serial sections of ovaries from PMSG-stimulated mice killed at different time points (18, 24, and 48 hours) after hCG administration. Note the invasive angiogenic process taking place during the entire time course, in which Dll4 staining (*green*) is mostly restricted to the leading edge and is mostly absent from the remaining areas of the growing vessel. In contrast, Notch-1 staining (*red*) is observed along the whole vascular vessel. This apparently mutually exclusive pattern for Dll4 and Notch-1 staining in the vascular sprout is maintained for the first 24 hours after hCG administration; however, Dll4 staining vanishes afterward coinciding with closure of the CL gap and ending of the invasive process. Nuclei have been stained (*blue*) to better locate the growing advance of the invading cells. Original magnification, ×100.

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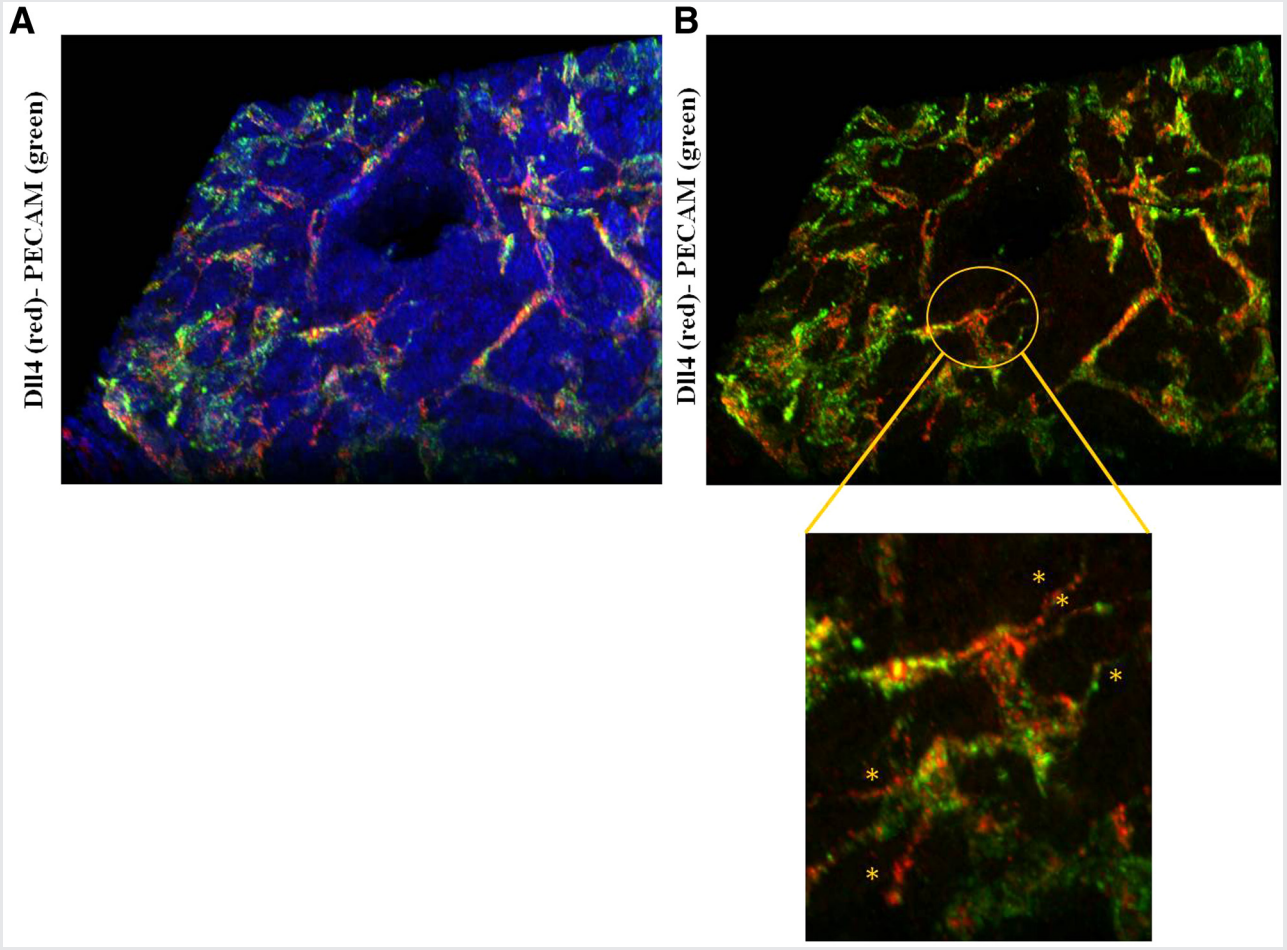
FIGURE 3



Identification of EC filopodia in the leading edge of the endothelial growth. (A) Images show double immunofluorescent detection of Dll4 (*red*) and PECAM (*green*), in thick ovarian sections from PMSG+hCG-stimulated mice (n = 3), 24 hours after hCG. Nuclei (*blue*) were stained with 6-diamino-2-phenylindole; areas in *yellow* represent overlapping of red and green signals. (B) Note the expression of Dll4 close to the not-yet-filled hole of the CL in formation, mostly on the edge of the invasive process taking place. Also note elongations, compatible with the existence of filopodia, on (Dll4-expressing) EC on the leading edge of growing neovessels (i.e., tip cells) (*asterisks*).

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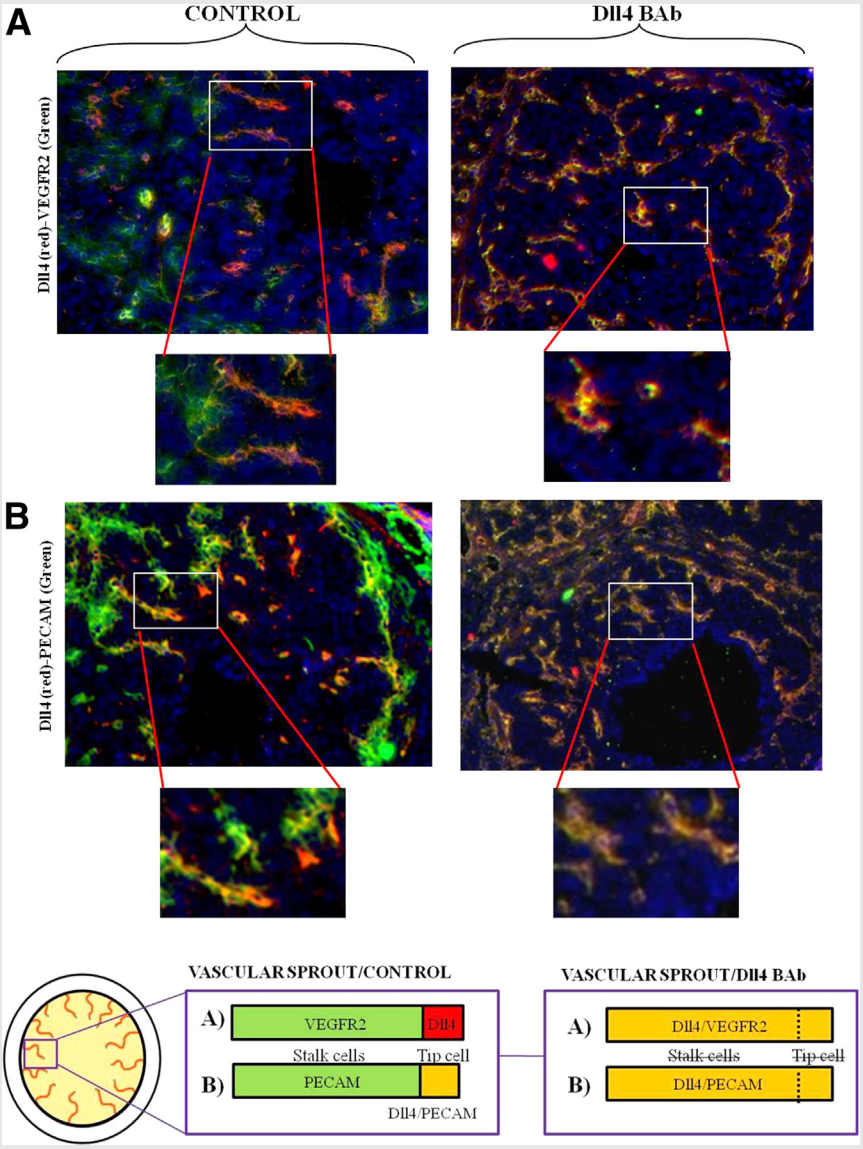
presented as mean ± SE. To compare sample means, ANOVA with Bonferroni correction and an unpaired Student's *t* test were performed. A *P* value of $<.05$ was considered a statistically significant difference.

RESULTS

Experiment 1: Identification of the Tip/Stalk Phenotype in CL Formation

Growing blood vessels were identified as semicontinuous longitudinal PECAM-positive structures "arising" perpendicularly from the circular theca layer, similar to spokes on a wheel (Fig. 1). Double immunofluorescence experiments in developing corpora lutea of mice suggested that the location of the Notch ligand Dll4 and its Notch-1 receptor is mainly restricted to vasculature, given that staining of both the ligand and receptor overlapped with that of PECAM (Fig. 2A and B). During the time course, Dll4 staining was restricted to the leading edge of the growing blood vessels and was mostly absent from other areas. In contrast, Notch-1 staining was observed along the whole vascular sprout but was largely absent in the leading edge areas, which were positive for Dll4 (Fig. 2C). This complementary and almost mutually exclusive Notch-1 and Dll4 expression pattern in the vascular sprout was maintained for the first 48 hours after hCG administration but not afterward (Fig. 2C). In addition to Dll4 expression, the cells in the leading edge of the growing vessels, which are supposedly tip cells, were also characterized by the presence of elongations that were consistent with the existence of filopodia at the leading edge of the blood vessels in the process of growth (Fig. 3 and Supplemental Video 1).

FIGURE 4



Effects of Dll4 blockade on morphologic features of the tip/stalk phenotype. Images show double immunofluorescent detection of Dll4 (*red*) in combination with (A) VEGFR2 and (B) PECAM (*green*) in the ovaries of PMSG+hCG-stimulated mice treated with an unspecific IgG or a Dll4 BAb and killed 24 hours after hCG administration. Overlapping between fluorescent signals is shown in *yellow*. An explanatory scheme has been added at the end of the figure to better illustrate the staining patterns observed in control and treated animals. Note how the pattern of Dll4 expression shifts from being mostly restricted to the leading edge of the growing vessels in the control group (unspecific IgG-treated) to promiscuous coexpression in experimental (Dll4 BAb-treated) animals. Original magnification, ×400.

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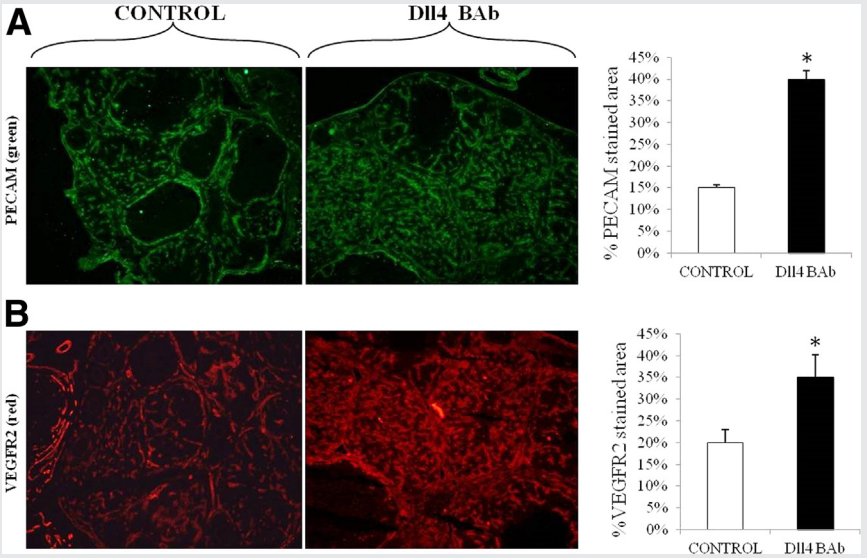
Experiment 2: Evaluation of the Effects of Dll4 Inhibition on the Morphologic Features of the Tip/Stalk Phenotype in CL

We subsequently studied the effects of the Dll4 BAb on the morphologic features of the luteal vasculature, which were identified in the previous experiments. Expression of Dll4 was mostly restricted to the tip of the blood vessels, in fact, coexpression of PECAM and Dll4 was mostly restricted to the leading edge of the growing vessels (Fig. 4B, control). The most dramatic and clear identification of the tip/stalk phenotype was observed when Dll4 and VEGFR2 staining was combined and a mutually exclusive pattern of expression in growing vessels was observed (Fig. 4, scheme). Indeed, Dll4 was present at the tip but not in the rest of the growing blood vessels, whereas VEGFR2 was absent from the tip and only present in the stalk cells of the blood vessels (Fig. 4A, control). Interestingly, the pattern of Dll4 expression shifted from being mostly restricted to the leading edge of the growing vessels in control groups (unspecific IgG-treated) to promiscuous coexpression in experimental (Dll4 BAb-treated) animals (Fig. 4A and B).

FIGURE 5



Effects of Dll4 blockade on morphologic features of the tip/stalk phenotype on luteal vasculature. (A) Illustrative staining pattern for PECAM (vascularization) and graph with quantitative analysis of PECAM luteal stained area. (B) Illustrative staining pattern for VEGFR2 (*red*) and graph with quantitative analysis of VEGFR2 luteal stained area in the ovaries of PMSG+hCG-stimulated mice treated with an unspecific IgG or a Dll4 BAb and killed 24 hours after hCG administration. Note a dramatic increase in luteal vascular density (B) and VEGFR2 (C) in Dll4 BAb-treated vs. unspecific IgG control animals. *$P<.05$. Original magnification, ×100.

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This altered expression pattern was associated with the appearance of thickened vasculature (Fig. 5A), which occupied a higher luteal area. Indeed, quantitative analysis of PECAM (Fig. 5A, graph) and VEGFR2 (Fig. 5B, graph) confirmed a two- to threefold increase in the luteal vascular density in Dll4 BAb-treated vs. unspecific IgG-treated control animals.

The similar number of corpora lutea counted on the contralateral ovaries of experimental subjects (9.2 ± 0.5) when compared with controls (8.7 ± 0.7) indicates that alteration of the morphologic features of the tip/stalk phenotype and increased luteal vascularization induced by inhibition of Dll4 signaling during the periovulatory phase did not affect ovulation rates or interfere with the formation of the CL structure.

Experiment 3: Evaluation of the Effects of Dll4 Inhibition on the Functionality of Luteal Vasculature and CL

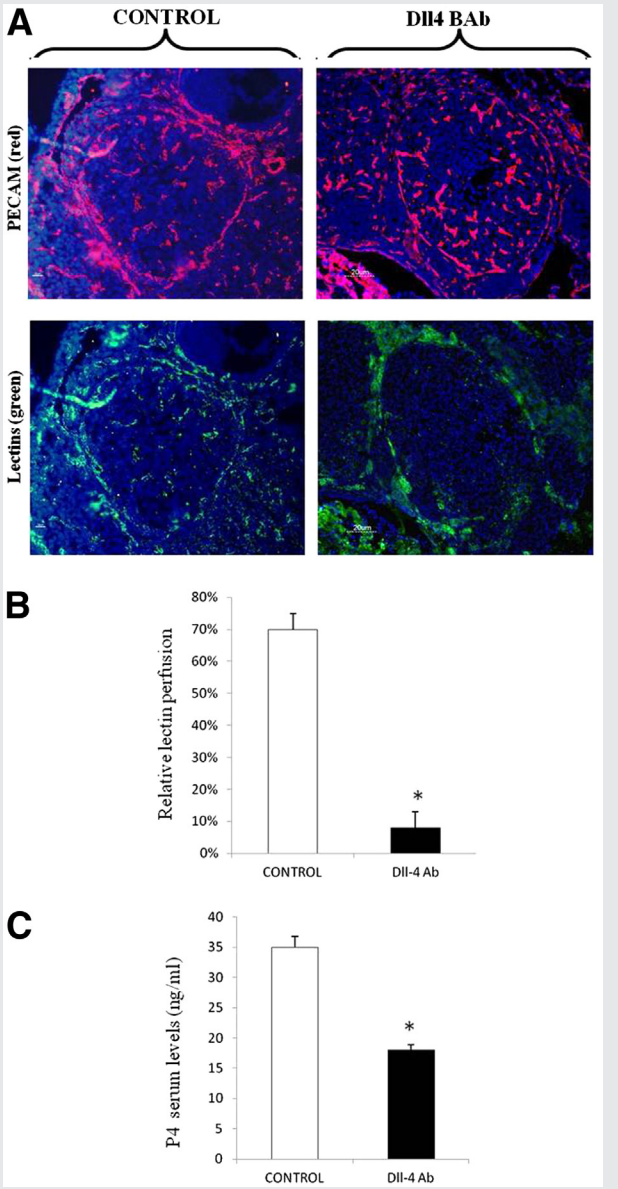
In our next set of experiments we assessed whether administration of a Dll4 BAb might alter vascular functionality, by determining the percentage of vessels stained with lectins. The rationale behind this is that EC-specific circulating lectins are only able to reach, bind, and stain adequately perfused (functional) vessels but not those with a defective lumen. We observed an increased amount of vascularization (PECAM-positive) unspotted with lectins in CL from Dll4-treated animals when compared with controls at 48 hours after hCG administration (Fig. 6B). This pattern was particularly evident in the inner central part of the completely formed CL of Dll4 BAb-treated animals. This contrasted with the pattern observed on IgG-treated animals, in which lectins seemed to homogenously stain the whole CL (Fig. 6A). The inability of lectins to reach the inner central part of mature CL suggests the presence of poorly perfused and thus dysfunctional vessels in Dll4-treated animals.

Analysis of structural luteolysis by evaluating the amount of apoptosis in the contralateral ovaries showed an apparent increase in this parameter in Dll4 BAb-treated animals; however, this increase was not statically significant (data not shown). In contrast, analysis of functional luteolysis by evaluating P serum levels revealed a clear and statically significant decrease in Dll4 BAb-treated vs. control animals (Fig. 6C). Despite P levels not dropping off dramatically, the presence of luteolytic effects suggests that alteration of vascular functionality induced by inhibition of Dll4 signaling was of sufficient magnitude to partially affect the functionality of the mouse CL.

DISCUSSION

Understanding how the complex reproductive angiogenesis process is regulated under physiologic conditions is essential to the correct treatment of related reproductive disorders. Vascular endothelial growth factor is known to play a key role in ovarian angiogenesis and function (3, 20), but the requirement for other factors in the tight regulation of the angiogenic process remains to be fully elucidated (21). In this article we have shown that VEGFR2-mediated sprouting and proliferation during luteal angiogenesis is modulated by the Dll4/Notch-1 pathway, which induces a tip/stalk phenotype.

FIGURE 6



Effect of Dll4 BAb on the vascular integrity and related function of the CL. (A) Illustrative images corresponding to the detection of vascular luteal functionality by complementary detection of intravital lectin perfusion (perfused vessels in *green*) and PECAM (total amount of blood vessels in *red*) in contiguous serial ovarian sections. (B, C) Graphs corresponding to (B) the analysis of vascular luteal functionality, as the percentage of PECAM-positive area spotted with lectins, and (C) luteal function as determined by the amount of circulating P in PMSG+hCG-stimulated mice treated with an unspecific IgG or a Dll4 BAb and killed 48 hours after hCG administration. Note in (A) that the completely formed CL in the control animals (*left*) show homogeneous lectin staining complementary to PECAM in the inner part, whereas the CL of Dll4 BAb-treated animals show a lack of lectin staining in the inner part of the CL despite the presence of PECAM-positive signal. Such a staining pattern is compatible with the presence of poorly perfused [as shown in (B)] and thus dysfunctional blood vessels leading to partial impairment of related function [note decreased P in (C)]. Original magnification, (A) ×400, (B, C) ×100. *$P<.05$.

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The tip/stalk hypothesis proposes that during VEGF/VEGFR2-initiated neovessel growth, there is EC cross-talk through the Dll4/Notch-1 pathway and that this determines which cells lead the advance of the vessel sprouting and which ones follow (22). Tip cells emit filopodia and specifically express Dll4, which activates Notch-1 in stalk cells; Notch-1 signaling abrogates Dll4 expression in the stalk. Therefore, by establishing a hierarchy with specific Dll4 expression in the tip, chaotic sprouting and growth is prevented (14).

In agreement with the tip/stalk hypothesis (14), ECs with filopodia and Dll4 expression were detected and were mostly restricted to the leading edge of the concentrically growing luteal vessels, thus shifting from peripheral to more central inner positions as the invasive process took place. Interestingly, Dll4 staining almost entirely disappeared in completely filled CL, thus accounting for extinction of tip/stalk regulation at the end of the invasive/angiogenic process.

Administration of anti-Dll4 BAb induced a dramatic alteration in the Dll4 expression pattern, which shifted from being mostly restricted to the leading edge of the growing blood vessel to being promiscuously coexpressed in all PECAM-positive cells, followed by a dramatic two- to three-fold increase in luteal vascular density in Dll4 BAb. These results are compatible with hierarchical tip/stalk regulation of neovessel growth during luteal angiogenesis and thus recapitulate previous findings in the retina model (14): loss of the tip/stalk phenotype due to deregulation of the Dll4/Notch-1 signaling feedback loop leads to hypersprouting and overproliferation of ECs.

In contrast to CL, follicular angiogenesis did not seem to be governed by induction of the tip/stalk phenotype. In fact we did not observe any changes in the number, size, or shape of CL, nor find any alterations in the theca layer vasculature and related ovulatory processes in Dll4 BAb-treated animals. These results suggest that induction of the tip/stalk phenotype is not likely to function in nonsprouting elongation angiogenesis phenomena such as those observed in the theca layer vessels during follicular development (23) or in the proliferative phase in the functional layer (24). On the basis of reports in murine tumor models, we predicted the enhanced neoformation of nonfunctional luteal vessels in the ovaries of our Dll4 Bab-treated mice. Therefore, we compared the functionality of luteal vessels in experimental vs. treated conditions by assessing the percentage of vessels spotted with specific lectins in completely developed CL. Whereas no apparent differences between groups were detected in the peripheral vessels, perfusion in the inner central part of CL in Dll4 BAb-treated animals was almost completely abrogated when compared with controls. Given that angiogenesis takes place radially from the periphery toward the inner central area (2), it is likely that structural alterations and chaotic growth induced by the anti-Dll4 BAb only became evident as the vessels grew and perfusion became worse.

Next we assessed whether Dll4 inhibition might result in the uncoupling of the conventional link between vascular development and luteal function (10, 25). Therefore we searched for the presence of structural or functional luteolysis, which may indicate such an effect. Given that

P levels were decreased by twofold in Dll4 BAb-treated mice, it is likely that luteal function was affected owing to alterations in the luteal vascular structure. However, because P levels did not drop dramatically it is plausible that luteal function was maintained to a certain degree because alterations in luteal vascular functionality induced by anti-Dll4 BAb do not limit O_2/nutrient delivery below that of passive diffusion/cell-to-cell communication. Alternatively, we speculate that luteal function might be maintained because of P production by luteal granulosa cells in the already well-irrigated periphery of CL, despite the death/apoptosis of the poorly irrigated luteal granulosa cells present in the inner part. Nevertheless, the former possibility is less likely, given the fact that the tendency to increased apoptosis observed in the inner central part of CL from experimental mice when compared with controls was not statically significant.

These data mostly agree with a recent report from Fraser et al. (25), which shows that anti-Dll4 BAb administration in marmosets induced structural defects in luteal vasculature and increased angiogenesis, a finding that is compatible with deregulation of the tip/stalk. However, and somehow different from our findings, this group observed a more dramatic decrease in P in Dll4 BAb-treated marmosets than we did, suggesting that luteal function had been completely impaired owing to defects in the vasculature in these animals. The differences in the intensity of the luteolytic effect accomplished between both works might be a result of the differences in the animal models used but also suggest that exclusive compensatory mechanisms exist in mice, which are able to rescue CL function from the devastating effects that Dll4 deregulation exerts in marmosets.

We are unaware of whether decreases in P levels observed in our Dll4-treated mice can interfere with related pregnancy. In the hypothetical event that the effects of Dll4 BAb on pregnancy were studied it would be difficult to dissect the effects caused by interference with CL function from those debts to alteration of angiogenesis in other reproductive organs. Indeed decidual and placental angiogenesis as well as embryo development are VEGF/VEGFR2 (9, 26) driven processes that consequently might also be susceptible to be regulated by the Dll4/Notch-1.

In conclusion, although the paradoxical effects of impaired luteal vascular functionality but not completely affected luteal function in response to Dll4 blockade in the mouse model remain to be elucidated, we have shown that VEGFR2-mediated sprouting and proliferation during luteal angiogenesis is modulated by the Dll4/Notch-1 pathway by inducing a tip/stalk phenotype.

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SUPPLEMENTAL METHODS

Histology and Immunofluorescence

Frozen ovaries were sliced into 8 μm sections at 50 μm intervals and mounted on Superfrost Gold glass slides (Thermo Fisher Scientific Inc.). Hematoxylin and eosin (H&E) staining was performed according to standard procedures (1). Serial sections were fixed in acetone at -20° C, before the immunostaining procedure was performed. Before applying the primary antibody, avidin-biotin-blocking solution (Vector laboratories) was used to prevent nonspecific staining, followed by incubation with a PBS (Sigma-Aldrich blocking solution containing 2% normal serum (Thermo Scientific) matching with the secondary antibody, and 5% BSA (Roche). Primary antibodies were used at the following dilutions: PECAM antibody (rat anti-mouse CD31 antibody, 1:50; BD Pharmingen), goat anti-mouse Notch-1 (1:200; R&D Systems Inc.), goat anti-mouse Dll4 (1:200; R&D Systems), and goat anti-mouse VEGFR-2 antibody (1:200; R&D Systems). We used mouse brain as a positive control for PECAM, Notch-1, and Dll4 antibodies (data not shown). Primary antibodies were incubated overnight at 4° C in a humidified chamber, while negative controls were left in the blocking solution. The sections were incubated with the specific secondary biotinylated antibody for each primary antibody (1:1000, Vector Laboratories) for 30 minutes at room temperature and signals were visualized with fluorescent dye conjugates of streptavidin: AlexaFluor488 and AlexaFluor594 (1:1500, Molecular Probes). In addition, to study co-expression, dual immunofluorescence of PECAM was performed in combination with Dll4, VEGFR-2, or Notch-1 antibodies. After staining for one antigen, avidin-biotin-blocking solution (Vector laboratories) was used to avoid nonspecific staining of the second antigen. Two sections for each embryonic day were analyzed, and five images were randomly obtained from each section with a fluorescence microscope (Nikon Eclipse E400; Nikon, Japan) connected to a digital camera (Nikon Digital Camera, Dxm1200F), and double images were created with Adobe Photoshop CS3 (Adobe Systems, Inc.).

3D Analyses of Corpus Luteum Vessels

Following immunofluorescent staining of control animal ovarian sections for the vessel marker PECAM and Dll4 ligand, FluoView software (Olympus, Japan) was used to reconstruct 3D images of the CL vessels from Z-section series obtained by confocal microscopy (FV-1000, Olympus) in order to identify EC filopodia at the leading edge of the endothelial growth in CL.

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