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Inhibition of Delta-like 4 mediated signaling induces abortion in mice due to deregulation of decidual angiogenesis☆

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ABSTRACT

Objective: To explore whether the Dll4/Notch1 pathway plays a key role in regulating the vascular endothelial growth factor (VEGF)/VEGF receptor 2 (VEGFR2) driven decidual angiogenesis and related pregnancy through induction of a tip/stalk phenotype.
Methods: Progesterone-replaced ovariectomized pregnant mice received a single injection of YW152F (Dll4 blocking antibody, BAb) or placebo at embryonic day (E) 4.5. Animals were sacrificed at different time points; blood and uterus were collected for further analysis. Number of embryos and implantation site, uteri weight, and serum progesterone levels were assessed. Alterations in the tip/stalk phenotype were determined by quantitative immunofluorescent analysis of vascularization, Dll4 expression, cellular proliferation and apoptosis in uterine sections.
Results: Abrogation of Dll4 signaling leads to a promiscuous expression of Dll4, increased cell proliferation, apoptosis and vascularization at E 6.5. Such an abrogation was associated with a dramatic disruption of embryo growth and development starting at E 9.5.
Discussion: The observed promiscuous expression of Dll4 and the increase in cell proliferation, apoptosis and vascularization are events compatible with loss of the tip/stalk phenotype. Excessive (although very likely defective) decidual angiogenesis due to such vascular alterations is the most likely cause of subsequent interruption of embryo development and related pregnancy in Dll4 treated mice.
Conclusions: Dll4 plays a key role in regulating decidual angiogenesis and related pregnancy through induction of a tip/stalk phenotype.

1. Introduction

Decidualization involves rapid proliferation and differentiation of uterine stromal cells, into the morphologically and functionally distinct new structure called decidua [1,2]: a pre-placental structure which is indispensable for early post-implantation embryo development [3] until active placental development occurs (E 9.5–10.5) [2,4]. During decidualization endothelial cells (ECs) proliferate, invade the stroma and form an extensive vascular network which supports the growth and subsequent maintenance of the decidua [5]. Impairment of decidual angiogenesis leads to termination of pregnancy thus showing a clear link between vascular development and decidual function [2,6]. Alterations of decidual angiogenesis contribute to several pregnancy complications, such as preeclampsia and intrauterine growth restriction (IUGR) and are thus believed to be important in the pathogenesis of idiopathic repetitive pregnancy loss [7]. Therefore, elucidating how decidual and subsequent placental microvasculature is regulated under physiological conditions is key to identifying gene targets that can be manipulated to try to attenuate the pathological situations mentioned above.

Previously, we [6] and others [2] demonstrated that mouse decidual angiogenesis and related pregnancy can be blocked by interference with the vascular endothelial growth factor (VEGF)/VEGF receptor 2 pathway (VEGFR2 pathway); the main player in

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initiating sprouting angiogenesis. Nevertheless, although VEGF is the principal stimulator of EC proliferation, the formation of the complex networks of blood vessels requires the interplay of various angiogenic factors in order to tightly regulate sprouting, guide vessel growth, and organize vessel assembly [8]. Among the many genes involved in angiogenesis, the interaction between the Dll4 ligand and the Notch1 receptor in adjacent ECs,[2] has been proposed to play a key role in regulating the bulk of sprouting angiogenesis initiated by VEGF/VEGFR2 by inducing a tip/stalk phenotype [9].

The tip/stalk hypothesis proposes the existence of specialized ECs, located at the tips of growing capillaries during neovascularization [10]. Unlike their neighboring stalk cells, which proliferate and form the vascular lumen, these endothelial tip cells do not undergo mitosis but rather initiate sprouting and migration thus guiding outgrowing capillaries [9]. During VEGF/VEGFR2-initiated neovessel growth, endothelial Dll4 expression, which is mostly restricted to tip cells, activates Notch1. Activation of Notch1 on stalk cells initiates a feedback-back loop which results in restriction of new sprout development by avoiding Dll4 expression in the stalk [10,11]. Therefore, by restricting Dll4 expression to the tip, EC acquire a hierarchy in the growing sprout guiding growth and preventing chaotic sprouting in response to VEGF/VEGFR2 [12]. In agreement with this hypothesis, the importance of the VEGF/VEGFR2–Dll4/Notch1 feedback loop in fine-tuning angiogenesis has been demonstrated in tissues undergoing physiological i.e. mouse retina [11–13] and pathological (i.e. tumor vessel) angiogenesis [14]. In these tissues, the systemic blockade of Dll4 paradoxically induces the overexpression of this ligand due to the fact that the pathway inhibiting the promiscuous expression of Dll4 in all EC is inhibited. Promiscuous Dll4 overexpression that is caused by inactivation of Notch1 signaling, leads to loss of hierarchic growth, and EC chaotically hyperproliferate resulting in an increased network of excessively-branched small interconnected vessels. These newly growing aberrant vessels are non-functional due to structural abnormalities caused by their chaotic growth and assembly. As a consequence of this the functionality of these structures is paradoxically compromised, i.e. tumor growth inhibited and corpus luteum (CL) undergoes luteolisis despite the presence of increased angiogenesis. This phenomenon is known as Dll4 paradox [14]. Based on the descriptive similarities of the VEGFR2-driven angiogenic events described here and the reported presence of some components of the Notch family in decidual blood vessels [14] and in early stages of placentation [15], we hypothesized that Dll4/Notch1 might play a key role in regulating decidual sprouting angiogenesis by inducing a tip/stalk phenotype during the peri-implantation period.

To test such hypothesis, we searched for morphological and functional features compatible with a tip/stalk phenotype. Morphologically, we sought for the existence of a polarized pattern of Dll4 expression restricted to the tip of decidual growing neovessels. Functionally, we aimed to study whether inhibition of Dll4 mimicked a Dll4 paradox pattern with affectation of related function. For functional purposes a single dose of YW152F (10 mg/kg) was employed [14]. YW152F is an anti-Dll4 human IgG blocking antibody (Dll4 BAb) which targets *in vivo*, the interaction of Dll4 with its Notch ligands. At a 10 mg/kg dose the biological action of YW152F lasts no more than 3 days and is effective in inhibiting tumor angiogenesis [16].

[2] Delta-like 4 (Dll4), Endothelial Cells (ECs), Intrauterine Growth Restriction (IUGR), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor (VEGFR2), corpus luteum (CL), Dll4 blocking antibody (Dll4 BAb), hematoxylin and eosin (H&E), progesterone-replaced ovariectomized pregnant (PROP) mice, embryonic day (E).

2. Materials and methods

2.1. Animals

Progesterone-replaced ovariectomized pregnant (PROP) mice [2,6] with time specific, systemic administration of the Dll4 BAb as is explained in Table 1 and described in detail in Supplemental Material and methods, were used. By doing so, we ensured that the effects of the Dll4 BAb were restricted to the maternal part of the pregnant uterus, specifically to decidual angiogenesis and not to interference with luteal support of pregnancy [17]. Such an experimental approach had been used successfully previously to study the role of the VEGF/VEGFR2 pathway in decidua of pregnant animals [2,6]. All of our *in vivo* studies were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Protocols for animal handling were approved by the animal ethics committee at the Valencia University School of Medicine.

2.2. Experimental design

PROP mice received a single 10 mg/kg i.p. injection of either an unspecified human IgG (control group; $N = 27$) or the anti-Dll4 BAb, YW152F (experimental group; $N = 27$) on embryonic day (E) 4.5. As the effects of such BAb last for about 3.5 days [14], antibody activity on E 8.5 would have been minimal or absent. To assess whether antibody injected on E 4.5 could be detected in embryos on E 8.5 we stained for the possible presence of this antibody (Supplemental material). We did not detect any antibody presence in E 8.5 embryos (Supplementary Fig 1). Animals were narcotized by i.p. injection of ketamine and xylazine (170–260 mg/kg and 8.6–13 mg/kg respectively) and blood was obtained by cardiopuncture immediately before sacrifice of mice on E 5.5 ($n = 3$), 6.5 ($n = 5$), 7.5 ($n = 5$), 8.5 ($n = 3$), 9.5 ($n = 5$), 11.5 ($n = 3$), and 13.5 ($n = 3$) in each group. Subsequently, the pregnant uterus was surgically removed intact, weighed and the implantation sites and embryos per uterus were counted by visual inspection. The collected uteri were embedded in OCT (optimal cutting temperature) medium and, stored at −80 °C for subsequent histological and immunohistochemical analysis.

2.2.1. Histological evaluation and immunofluorescent analysis of vascularization, cellular proliferation and Dll4 expression

Frozen uteri were sliced into 8 μm sections at 48 μm intervals and mounted on Superfrost Gold glass slides (Thermo Fisher Scientific Inc., Waltham, MA). Every 10th to 15th sections were stained with hematoxylin and eosin (H&E) to detect the sections containing the structures of interest [18]. Next, selected sections were fixed in acetone at −20 °C, and immunofluorescence analysis for PECAM (CD31, a specific vascularization marker), Ki67 (a marker of cell proliferation) and Dll4 was performed as described in detail in Supplementary Methods.

2.2.2. Apoptosis detection

Apoptotic cells were detected by TUNEL, using an ApopTag ISOL Dual Fluorescence Apoptosis Detection Kit (DNase types I & II; Millipore, Billerica, MA), according to the manufacturer's instructions.

2.2.3. Serum progesterone (P4) levels

Serum P4 levels were measured using a competitive chemiluminescent immunoassay (Diagnostic Products Corp./Siemens, Los Angeles, CA). The intra- and interassay coefficients of variation were 5.2 and 6.7%, respectively.

2.2.4. Data analysis

Quantitative analysis of vascularization (CD31 expression), Dll4 expression, cellular proliferation (Ki67 expression), and apoptosis (Tdt-OH expression) was performed using an image analysis system linked to a Nikon Eclipse E400 microscope (Nikon, Japan), followed by analysis with Image pro plus (Media cybernetics, Warrendale, PA) and Image J software as previously described [19]. Four random power fields of similar regions of the decidua were photographed per each conceptus and at least three conceptuses from each animal were employed for the determination of each of the parameters of interest mentioned below. In previous studies published by our group [6] we observed that secondary antibodies tend to generate a non-specific signal in the area surrounding the embryo. Hence, in order to avoid over-estimation of both, Dll4 and PECAM expression, the intense signal surrounding the embryo was not considered, neither in control and treated animals.

Table 1
Experimental design for P4 replaced-ovariectomized pregnant mice (PROP mice).

	Mate mice 5–11 pm
E 0.5	Confirm pregnancy
E 4.5 + 2 h	P4 replacement
E 0.5 + 6 h	Bilateral ovariectomy
E 0.5 + 7 h	Inject Dll4 blocking antibody