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Vascular Endothelial Growth Factor (VEGF) Isoforms may Regulate Sex-Specific Vascular Development, Cord Formation and Follicle Progression in Developing Gonads

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1 **VASCULAR ENDOTHELIAL GROWTH**
2 **FACTOR (VEGF) ISOFORMS MAY REGULATE**
3 **SEX-SPECIFIC VASCULAR DEVELOPMENT,**
4 **CORD FORMATION AND FOLLICLE**
5 **PROGRESSION IN DEVELOPING GONADS**

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9 **Summary**

10 The ratio of VEGF angiogenic to anti-angiogenic isoforms appears
11 to determine different biological functions in reproduction. Reduced
12 amounts of angiogenic VEGF isoforms inhibit testis sex-specific
13 vasculature and normal cord formation in organ cultures while reduction
14 of inhibitory isoforms increased vasculature and perturbed cords. In the
15 female, using peri-natal ovarian cultures, inhibition of angiogenic
16 VEGF isoforms reduced vascular development and inhibited follicle
17 progression while conversely reductions in inhibitory isoforms or
18 increases in angiogenic isoforms enhanced follicle development. Thus,
19 regulation of the *Vegfa* gene to produce angiogenic or anti-angiogenic
20 isoforms may be a mechanism to alter sex-specific vascular development,
21 formation of seminiferous cords, and/or follicle progression within
22 mammalian gonads.

23 **Introduction**

24 Infertility affects 40-70 million couples; of those approximately
25 50% of the infertility problems are attributed to male-related factors which
26 include: low sperm count, abnormal spermatogenesis, and reduced
27 androgen production [1, 2]. Formation of testicular cords, and sex-
28 specific vasculature, are the two morphological hallmarks that distinguish
29 a testis from an ovary. Female-related infertility factors include: ovulatory
30 disorders, anovulation due to polycystic ovarian disease and ovarian
31 hyperstimulation syndrome, or premature ovarian failure. Some or all of
32 these female infertility problems may be caused by improper prenatal
33 development of the fetal gonad, reduction of number of primordial
34 follicles or disruption of progression of folliculogenesis.
35 Neovascularization of the ovary and continued formation of follicle
36 vasculature are critical events in normal reproductive function.

37 The VEGF family is composed of five ligands: VEGF (VEGF-A),
38 VEGF-B, VEGF-C, VEGF-D and Placenta Growth Factor. VEGF
39 (VEGF-A) is the best characterized and most potent VEGF molecule.
40 VEGF works through both Fms-like tyrosine kinase 1 (FLT1) and Kinase

41 domain region receptor (KDR), to elicit its effects on endothelial cell
42 migration, differentiation, proliferation and survival and apoptosis. VEGF
43 is transcribed from a single gene that has 8 exons and is alternatively
44 spliced into different isoforms each containing a different number of
45 amino acids. The most common angiogenic isoforms are VEGF205, 188,
46 164, 144, and 120 [3].

47 In 2002, an additional isoform, VEGF164b, was identified which
48 contained part of the 3' UTR that is now determined to be exon 8b.
49 Furthermore, recent studies have demonstrated that the human VEGF165b
50 isoform is anti-angiogenic in function and inhibits signal transduction
51 through KDR [4, 5]. Thus, this isoform is inhibitory to the actions of
52 VEGF. Therefore, it appears that for every angiogenic isoform there is a
53 sister inhibitory isoform that is formed when exon 8a is replaced with
54 exon 8b. These inhibitory (anti-angiogenic) isoforms serves to modulate
55 the functions of the angiogenic VEGF isoforms.

56 **Materials and Methods**

57 *Rat Testis Organ cultures:* E13 testes with attached mesonephros
58 were placed on Millicell CM filters (Millipore, Bedford MA) in drops of
59 medium floating on the surface of 0.4 ml of CMRL 1066 media (Gibco
60 BRL, Gaithersburg, MD) at conditions reported [6, 7]. One organ from
61 each animal was designated as a vehicle control, while its pair was
62 subjected to a VEGF receptor signal transduction inhibitor, VEGFR-TKI
63 (8 μ M), or a VEGF antagonist, Je-11 (10 μ g/ml) [8]. *Whole-mount IHC
64 and Confocal Microscopy:* After culture, the organs were fixed in 4%
65 paraformaldehyde. Samples were washed, blocked and whole-mount IHC
66 was conducted as reported [8]. *Vascular Density Quantification was
67 conducted as reported* using the staining index in Scion Image [8].
68 *Ovarian Organ Cultures:* Ovaries were dissected from postnatal day 3 and
69 4 (P3/4) rats (day of birth was considered to be P0). One ovary from each
70 animal was designated as a control, while its pair was subjected to
71 Treatment with 8 μ M VEGFR-TKI; Calbiochem, La Jolla, CA or KDR
72 signal transduction inhibitor, V1, (30 μ M; Calbiochem, La Jolla, CA),
73 VEGFA164 (R & D Systems Inc., Minneapolis, MN) or VEGF165b
74 antibody (5ng/ml or 50ng/ml) (Abcam, Cambridge, MA). All of these
75 treatments were added daily to the culture medium of the treated wells.

76 **Results**

77 Treatment of testis organ cultures with tyrosine kinase inhibitors to
78 the VEGF receptor signal transduction pathway (VEGFR-TKI) or to
79 VEGF antagonists (Je11) disrupted both sex-specific vascular
80 development and seminiferous cord formation. Vascular density was
81 reduced by 90 and 46%, respectively (P < 0.01). Conversely, treatment

82 with VEGF angiogenic isoforms: VEGF164, VEGF120 or an antibody to
83 the exon 8b which binds inhibitory isoforms increased vascular density
84 50-100% over controls and resulted in swollen and perturbed testis cord
85 formation. Thus angiogenic VEGF isoforms are important in establishing
86 the sex-specific vascular development and too much inhibitory isoforms
87 may alter the ability for this vasculature and subsequent testis cord
88 formation to occur.

89 In the female, treatment of perinatal rat ovaries signal transduction
90 inhibitors (VEGFR-TKI), antagonists to KDR (V1) arrested follicle
91 development to later secondary follicle stages ($P < 0.05$). In contrast,
92 treatment with angiogenic VEGF isoforms or an antibody to inhibitory
93 isoforms increased vascular development and accelerated follicle
94 progression to later secondary follicle stages ($P < 0.05$). Thus, we propose
95 that amount of angiogenic to inhibitory VEGF isoforms modulates follicle
96 progression and may determine whether an ovarian follicles continues to
97 progress or undergoes atresia.

98 **Conclusion**

99 Approximately two million couples seek treatment for infertility
100 every year and less than half find successful treatments [9, 10]. Infertility
101 problems in at least half of these couples are a result of male-related
102 factors that are created by testicular dysgenesis. Many of the problems
103 associated with testicular dysgenesis are proposed to involve a disruption
104 in embryonic differentiation of cells within the indifferent gonad resulting
105 in altered testicular development. Elucidating the factors involved in sex-
106 specific vascular development will allow for a better understanding of how
107 transcription factors coordinate regulation of growth factors to result in a
108 testis-specific vascular system. Furthermore, delineating the interaction of
109 VEGF angiogenic and inhibitory isoforms in ovarian follicle arrest and or
110 progression may also be an interesting piece in the puzzle of disorders
111 that result in female infertility.

112

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