VEGFR2 Expression Is Differently Modulated by Parity and Nulliparity in Mouse Ovary

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1. Introduction

A significant decline of fertility rate is occurring in developed countries, mainly due to economic problems and lifestyle choices. Therefore, many young women postpone pregnancy at older ages, often ignoring that quantity and quality of oocytes both decrease from 35 years onward [1, 2]. In addition, young women can experience fertility problems because of several pathological conditions affecting the ovary, first of all cancer [3–5], thereby increasing the number of childless women.

A key question is whether infertility and, even more, nulliparity can be considered as risk factors for ovarian cancer (OC) onset [3, 6, 7]. As a matter of fact, the risk seems to be increased also in women affected by polycystic ovarian syndrome (PCOS) [8], corpus luteum insufficiency [9], and endometriosis [10]. In women undergoing stimulation protocols during IVF procedures the question is still debated [11, 12]. Interestingly, all these diseases are characterized at the molecular level by elevated hypoxia and overexpression of proangiogenic factors, especially vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor type 2 (VEGFR2) [8, 13–15]. This is not surprising, as in the adult mammalian ovary a delicate balancing of pro/antiangiogenic factors participates in the physiological modulation of cyclical angiogenesis and vascular regression [8, 16–19].

To date, many efforts aimed at demonstrating the link between female infertility and increased OC risk have led to un conclusive results. It is noteworthy that, in mice, a full-term pregnancy significantly reduces the risk of developing chemically induced mammary cancers, compared with
nulliparous animals [20, 21]. In women, a full-term pregnancy seems to reduce the risk of developing different reproductive cancers, such as breast [22], ovarian [23], and endometrial cancer [24], compared with nulliparity [6, 25]. However, such a protective effect seems to be exerted only when the first full-term pregnancy occurs before 30 years of age [26].

The present study explored how pregnancy can exert its protective effects by assessing if VEGF and VEGFR2 expression were differently modulated in the ovaries of adult primiparous (mothers, M), compared with nulliparous (virgins, V) mice.

2. Materials and Methods

2.1. Chemicals. The chemicals used were purchased from the following sources: rabbit polyclonal VEGFA (sc-507) and phospho-ERK1/2 (Thr202/Tyr204; sc-16982-R); mouse monoclonal Flk1 (sc-6251; VEGFR2), ERK1/2 (sc-153900), and α/β tubulin (sc-51502); goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (sc-2004) and goat anti-mouse IgG conjugated to HRP (sc-2005) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit monoclonal phospho-VEGFR-2 (pY1173; #2478), rabbit polyclonal phospho-PLCγ1 (Tyr783; #2821), and PLCγ1 (#2822) were purchased from Cell Signaling Technology (Beverly, MA, USA). SuperSignal West Pico Chemiluminescent substrate (34080) and RNAlater™ Stabilization Solution (AM7020) were purchased from Thermo Scientific (Rockford, IL, USA); RNeasy Mini Kit was purchased from Qiagen (Chatsworth, CA); ThermoScript™ RT-PCR Transcription kit was purchased from Invitrogen (Milan, Italy); PowerUp SYBR™ Green Master Mix was purchased from Life Technologies (Carlsbad, CA, USA). Primer sequences were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Eosin–Floxin alcoholic solution (05-10020/L) and Carazzi’s Hematoxylin–Nuclear staining (05-06012/L) were purchased from Bio-Optica (Bio-Optica Milano SpA, Milan, Italy). All other reagents were obtained from Sigma-Aldrich Company (St. Louis, MO, USA).

2.2. Animals and Ethical Approval. Mus Musculus Swiss CD1 female mice (Harlan Italy, Udine, Italy) were housed in an animal facility under controlled temperature (21±1°C) and light (12 h light/day) conditions, with free access to food and water. Mice of the same 2 months old, n = 40) were sorted into 2 groups: (a) mothers (M, n = 20) that were mated with males of proven fertility and (b) virgins (V, n = 20), unmated, that were kept alone. Once the pregnancy was established, the group of M mice were kept alone until the birth of offspring and weaning. Afterwards, M and V mice (4 months old) were sacrificed at the postovulatory stage of estrus, determined by the analysis of vaginal smears [27]. After collection, ovaries were snap frozen and stored at −80°C for western blotting and histological analysis or for quantitative real-time PCR (qrt-pcr) in RNAlater Stabilization Solution. When utilized for molecular analyses, ovaries were processed using a rotor-stator tissue homogenizer (Precellys 24, Bertin Technologies) for two cycles of 10 s at 5000 x g.

All experimental procedures involving animals and their care were performed in conformity with national and international laws and policies (European Economic Community Council Directive 86/609, OJ 358, 1 Dec 12, 1987; Italian Legislative Decree 116/92, Gazzetta Ufficiale della Repubblica Italiana n. 40, Feb 18, 1992; National Institutes of Health Guide for the Care and Use of Laboratory Animals, NIH publication no. 85-23, 1985). The project was approved by the Italian Ministry of Health and the internal Committee of the University of L’Aquila. All efforts were made to minimize suffering.

2.3. Somatic Cells Retrieval. Corpus luteum tissues (CLT) were obtained as described by Park et al. [28]. Briefly, CLT were dissected from ovaries under light field stereoscope and separated from the residual stromal tissues (RST). Samples were immediately snap frozen at −80°C for western blotting or stored at −80°C for RT-PCR in RNAlater Stabilization Solution.

2.4. H&E Staining. Ovaries were processed and stained according to the protocol of Park et al. [28]. After fixation overnight (o.n.) in 4% formalin, ovaries were embedded in paraffin, sectioned (5 μm/section), stained, and mounted. Sections were examined using StereoZoom® Leica S8 APO and images were acquired with Leica EC3 camera.

2.5. RNA Isolation and Relative Real-Time PCR. Total RNA was extracted from each sample using the RNeasy Mini Kit, according to manufacturers’ protocols. Quality and quantity of extracted RNA were measured by calculation of the optical density with an ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE).

In order to detect mRNA expression levels of genes of interest, qrt-pcr was performed. Briefly, using mRNA as template, single-stranded cDNAs were generated from 500 ng of total RNA by the ThermoScript™ RT-PCR Transcription kit (Invitrogen, Milan, Italy) according to manufacturer’s directions. Vegf 120, Vegf 164, and Vegfr2 levels were measured by qrt-pcr on the 7500 Fast real-time PCR system with SYBR® Green Technology using 100 ng cDNA mixed to 20 μl of PowerUp SYBR® Green Master Mix (Life technologies) and 500 nM of each forward and reverse primer (Table 1). The thermal cycling conditions were as follows: 2 min at 50°C and 2 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Levels of gene expression were reported as relative units with respect to mRNA levels of Actin gene used as reference gene to normalize each sample, as previously published [29, 30]. Each gene was analyzed in triplicate. Relative quantitative evaluation of mRNAs was performed by comparative ΔΔCt method.

2.6. Western Blotting. Samples were resuspended in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Igepal) containing protease inhibitors (1 mM phenylmethylsulphonylfluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and phosphatase inhibitors (1 mM sodium fluoride, 10 mM sodium pyrophosphate, and 1 mM sodium...
### Table 1: Primer sequences for RT-PCR. Note: R1: reverse; F1: forward.

| Oligo name          | Gene target | Sequence (5’-3’)            |
|---------------------|-------------|-----------------------------|
| mbeta actin R1      | Actin       | 5’-TGGACAGTGAGCCAGGATG-3’    |
| mbeta actin F1      | Actin       | 5’-TGTTGGTGCATAAAGAGAG-3’    |
| mFlk1 R1            | Vegfr2      | 5’-GACAGAAGGGATGATGGTG-3’    |
| mFlk1 F1            | Vegfr2      | 5’-GAGAGCAAGGCGCTGCTAGC-3’   |
| mVEGF 120 R1        | Vegf 120    | 5’-CGGCTTTGTCAATTTTCTGCG-3’  |
| mVEGF 120 F1        | Vegf 120    | 5’-GAGGTCCCATGAAATGGATCAG-3’ |
| mVEGF 164 R1        | Vegf 164    | 5’-AAAAAGCAGTAGTGATTTTCTGGC-3’|
| mVEGF 164 F1        | Vegf 164    | 5’-GAAGTCCCATGAAATGGATCAG-3’ |

![Figure 1: Quantification of corpora lutea number (CL) by H&E staining. Mother: M; virgin: V. In the representative images of ovaries from M (a) and V (b) mice, CLs were indicated by (+). Data from 4 different experiments are expressed as the mean ± SEM (c). Bar=1 mm.](image_url)

Orthovanadate, homogenized and centrifuged. Protein concentration was determined by Bio-Rad Protein Assay. Sixty μg of protein/sample was loaded onto 8% or 12% gels under reducing conditions, except for the VEGFR2 examined in nonreducing condition. After transfer, blots were incubated with anti-VEGFA (1:200), anti-pERK (1:200), anti-ERK (1:200), anti-pPLCγ1 (1:1000), anti-PLCγ1 (1:1000), anti-VEGFR2 (1:200), and anti-pVEGFR2 (1:1000) antibodies o.n. at 4°C.

HRP-conjugated goat anti-rabbit IgG (1:5000) and goat anti-mouse IgG (1:5000) were used as secondary antibody (1h, room temperature), and peroxidase activity was detected using a SuperSignal West Pico Chemiluminescent substrate. The nitrocellulose membranes were examined using the Alliance LD2-77WL imaging system (Uvitec, Cambridge, UK). Densitometric quantification was performed with the public-domain software NIH Image V.1.62 and standardized using tubulin as loading control. pVEGFR2, pERK, and pPLCγ1 signals were normalized to the respective total of VEGFR2, ERK, and PLCγ1, as previously described [31].

2.7. Statistical Analysis. All experiments were performed at least three times, and data were expressed as mean percentage ± SEM. Data from V mice were compared to data obtained from M mice, which were arbitrarily set as 100%. Also data from RST were compared to CLT, which were arbitrarily set as 100%. Experimental results of molecular analysis were analyzed using Student’s t-test. Results were considered statistically significant when \( P < 0.05 \). All statistical analysis was performed using the statistical package SPSS13.0 (SPSS Incorporated, Chicago).

3. Results

3.1. Histological Analysis. The analysis of ovarian morphology was performed to confirm that both parous (Mothers, M) and nulliparous (Virgins, V) mice were at postovulatory stage of estrus cycle [27]. The ovaries of both M and V mice showed similar morphological characteristics and number of corpora lutea (CL) (Figure 1; M versus V, \( P > 0.05 \)).

3.2. qRT-PCR. Analysis of Vegfa 164 and 120 isoforms of Vegfr2 transcripts were reported in Figure 2. In whole ovaries, mRNAs were expressed similarly between M and V mice (Figure 2(a); \( P > 0.05 \)). Even though Vegf 164 mRNA levels were slightly lower, there was no statistical difference in comparison with Vegf 120 levels (\( P > 0.05 \)). Concerning ovarian mRNA distribution, results show that both Vegfa isoforms and Vegfr2 mRNA were preferentially accumulated in corpus luteum tissues (CLT) rather than residual stromal tissues (RST) (Figure 2(b); \( P < 0.05 \)).
3.3. Western Blot Analysis. In whole ovaries, levels of VEGFA 120 and 164 isoforms were comparable between V and M mice (Figure 3(a); \( P > 0.05 \)), with a clear predominant expression of isoform 120 (almost 2-fold more than 164; \( P < 0.05 \)). As reported in Figure 3(b), both VEGFA 120 and 164 were more abundantly expressed in CLT than RST (Figure 3(b); \( P < 0.05 \)).

VEGFR2 quantification disclosed that the receptor was more accumulated in ovaries of V than M mice (Figure 4(a), upper and middle panel; \( P < 0.05 \)) and more predominantly in CLT (Figure 4(b), upper and middle panel; CLT versus RST, \( P < 0.05 \)). With respect to 1173-Tyr phosphorylation, the ratio pVEGFR2/VEGFR2 recorded in V ovaries decreased
Figure 3: Representative images and protein quantification of VEGF 164 and VEGF 120. Mother: M; virgin: V; corpus luteum tissue: CLT; residual stromal tissue: RST. Levels of VEGF 164 (upper and middle panel) and VEGF 120 (upper and lower panel) in whole ovaries of M and V mice (a) and their distribution in CLT and RST (b). As shown by the representative images (a, upper panel), ovarian VEGF 120 content is almost 2-fold more than VEGFA 164 (P < 0.05). Bar graph data represent the mean percentage ± SEM of 4 independent determinations. Data from V mice are compared to data obtained from M mice, which are arbitrarily represented as 100%. Data from RST are compared to CLT, which are arbitrarily represented as 100%. Comparisons are made within the same blot and across different blots. * P < 0.05.

4. Discussion

This study demonstrates that VEGFR2 protein and phospho-protein levels are differently modulated in ovaries collected from primiparous (M) and nulliparous (V) mice. Indeed, any parity-dependent effects on Vegfa (either 120 or 164) and Vegfr2 gene expressions were excluded because of comparable mRNA levels detected in the ovaries of both groups. Moreover, in agreement with literature data obtained on virgin animals [32, 33], also in M mice the expression of Vegf and Vegfr2 mRNAs occurs predominantly in CLT. Our results confirm that in ovaries of mice and large mammals, the predominant Vegf isoforms are 120 and 164 [29, 30], whereas proangiogenic isoform 144 is expressed at very low level and 188 is completely absent in mice [29].

At protein level, our results show that even if ovarian VEGFA 120 protein is more expressed than the 164 form, their respective contents in the ovaries of M and V mice are comparable. Both proteins are preferentially present in corpus luteum tissues (CLT), thus mirroring mRNAs distribution. We do not know why ovarian VEGFA 120 and
I64 protein contents differ, nor which of the two isoforms has a prominent role in our mouse model. As a matter of fact, also in other tissues and organs the basic mechanisms by which the different VEGFA isoforms operate are yet to be defined [34].

Although VEGFA 120 and 164 contents are significantly higher in corpus luteum tissue (CLT) of V mice in comparison with M mice, both VEGF isoforms are much more expressed in the residual stromal tissues (RST) of M than V mice. These findings suggest that pregnancy could more efficiently regulate follicle maturation, as already demonstrated in non-human primates and rodents [35, 36].

According to available human and rodent data [13, 37], also in our experiments VEGFR2 has been detected mainly in CLT, thus supporting the hypothesis that some cells of granulosa-lutein tissue could act as an endothelial-like cell population [13]. Despite similar mRNA expression, we found that ovarian VEGFR2 protein is more accumulated in V than M mice. This indicates that a parity/nulliparity-dependent regulation of receptor stability/degradation could occur. Literature data show that the dysregulation of the synthesis/degradation of other pro/antiangiogenic factors, and/or of the transcription factor hypoxia inducible factor-1 alpha (HIF-1α) could affect the expression and activation of this receptor [9, 19]. In our model, a different reactivity to hypoxic stress has been ruled out because of similar levels of Vegf/Vegfr2 mRNA (present results) and of HIF-1α protein in both M and V mice (our unpublished data). Unfortunately, many of the events are involved in VEGFR endocytosis/trafficking and ubiquitylation, and also details
of signaling pathways are not completely understood, and further studies are necessary to clarify all these issues [38]. However, it is generally accepted that low VEGFR2 levels are normally detected in adult vasculature and that the expression of this receptor is upregulated during inflammation or tumor growth [39].

The assessment of phosphorylation of VEGFR2 at Tyr1173, that is, the major proangiogenic signal for the induction of endothelial cell proliferation [9], reveals that only a fraction (~50%) of total receptor is phosphorylated in the ovaries of V mice in comparison with M mice, where almost all receptor (~90%) is phosphorylated. However, since V mice used in present experiments are healthy and young and therefore potentially fertile, it is not surprising that dVEGFR2 content and distribution in CLT and RST of V mice are similar to those recorded in M animals. From literature data, the overactivation of VEGFR2 represents a negative event in the generation of a pathological proangiogenic environment [40, 41]. Therefore, we can speculate that phosphorylation of all receptor molecules could have more dramatic effects in nulliparous than in parous mice, because of a potentially stronger proangiogenic stimulus.

Among the various phosphotyrosine residues, it seems noteworthy that phosphorylation of Tyr1173 stimulates the activation of phospholipase C$\gamma$ (PLC$\gamma$-) dependent proangiogenic ERK pathways [38]. When we analyzed these proteins in our samples, we found low signals in both M and V ovaries, thereby supporting the hypothesis that VEGFR2 signaling is not altered in the ovaries of M and V mice and that a yet unknown mechanism prevents complete VEGFR2 phosphorylation in V ovaries.

5. Conclusion

In conclusion, we suggest that nulliparity and parity differently modulate ovarian VEGFA/VEGFR2 system and that more favorable conditions to potentially altered ovarian angiogenesis more likely occur in nulliparous than in parous females. Our results could, at least in part, help to explain epidemiological data showing that OC incidence is significantly higher in infertile women and especially in nuns [25].

Data Availability

Molecular data used to support the findings of this study are included within the article and available from the corresponding author upon request.

Disclosure

The authors declare that an earlier version of this work has been presented as an abstract to the 5th World Congress of the International Society for Fertility Preservation (ISFP).

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors’ Contributions

Valentina Di Nisio and Gianna Rossi equally contributed to this paper.

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References

[1] Z.-J. Ge, H. Schatten, C.-L. Zhang, and Q.-Y. Sun, “Oocyte ageing and epigenetics,” Reproduction, vol. 149, no. 3, pp. R103–R114, 2015.
[2] K. E. Liu and A. Case, “No. 346-Advanced Reproductive Age and Fertility,” Journal of Obstetrics and Gynaecology Canada, vol. 39, no. 8, pp. 685–695, 2017.
[3] I. Cetin, V. Cozzi, and P. Antonazzo, “Infertility as a Cancer Risk Factor - A Review,” Placenta, vol. 29, no. 2, pp. 169–177, 2008.
[4] N. Mahajan, “Fertility preservation in female cancer patients: An overview,” Journal of Human Reproductive Sciences, vol. 8, no. 1, pp. 3–12, 2015.
[5] K. E. Waimey, B. M. Smith, R. Confino, J. S. Jerrus, and M. E. Pavone, “Understanding Fertility in Young Female Cancer Patients,” Journal of Women’s Health, vol. 24, no. 10, pp. 812–818, 2015.
[6] B. Hanson, E. Johnstone, J. Dorais, B. Silver, C. M. Peterson, and J. Hotaling, “Female infertility, infertility-associated diagnoses, and comorbidities: a review,” Journal of Assisted Reproduction and Genetics, vol. 34, no. 2, pp. 167–177, 2017.
[7] S. Ramakrishnan, I. V. Subramanian, Y. Yokoyama, and M. Geller, “Angiogenesis in normal and neoplastic ovaries,” Angiogenesis, vol. 8, no. 2, pp. 169–182, 2005.
[8] W. C. Duncan and J. Nio-Kobayashi, “Targeting angiogenesis in the pathological ovary,” Reproduction, Fertility and Development, vol. 25, no. 2, pp. 362–371, 2013.
[9] M. Shibuya, “Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases,” The Journal of Biochemistry, vol. 153, no. 1, pp. 13–19, 2013.
[10] M. Kva$\text{sk}$off, F. Mu, K. L. Terry et al., “Endometriosis: A high-risk population for major chronic diseases?” Human Reproduction Update, vol. 21, no. 4, pp. 500–516, 2015.
[11] S. M. Bjornholt, S. K. Kjaer, T. S. S. Nielsen, and A. Jensen, “Risk for borderline ovarian tumours after exposure to fertility drugs: Results of a population-based cohort study,” Human Reproduction, vol. 30, no. 1, pp. 222–231, 2015.
[12] M. M. Reigstad, I. K. Larsen, T. Á. Myklebust et al., “Cancer risk among parous women following assisted reproductive technology,” Human Reproduction, vol. 30, no. 8, pp. 1952–1963, 2015.
[13] M. Antczak and J. Van Blerkom, “The vascular character of ovarian follicular granulosa cells: Phenotypic and functional
evidence for an endothelial-like cell population,” Human Reproduction, vol. 15, no. 11, pp. 2306–2318, 2000.

[14] I. Sher, S. A. Adham, J. Petrik, and B. L. Coomber, “Autocrine VEGF-A/KDR loop protects epithelial ovarian carcinoma cells from anoikis,” International Journal of Cancer, vol. 124, no. 3, pp. 553–561, 2009.

[15] W. A. Spannuth, A. M. Nick, N. B. Jennings et al., “Functional significance of VEGFR-2 on ovarian cancer cells,” International Journal of Cancer, vol. 124, no. 5, pp. 1045–1053, 2009.

[16] V. R. Araújo, A. B. G. Duarte, J. B. Bruno, C. A. Pinho Lopes, and J. R. De Figueiredo, “Importance of vascular endothelial growth factor (VEGF) in ovarian physiology of mammals,” Zygot
e, vol. 21, no. 3, pp. 295–304, 2013.

[17] G. Macchiarelli, M. G. Palmerini, S. A. Nottola, S. Cecconi, K. Tanemura, and E. Sato, “Restoration of corpus luteum angiogenesis in immature hypothyroid rdw rats after thyroxine treatment: Morphologic and molecular evidence,” Theriogenology, vol. 79, no. 1, pp. 116–126, 2013.

[18] J.-Y. Jiang, K. Miyabayashi, S. A. Nottola et al., “Thyroxine treatment stimulated ovarian follicular angiogenesis in immature hypothyroid rats,” Histology and Histopathology, vol. 23, no. 11, pp. 1387–1398, 2008.

[19] C. Rico, A. Dodelet-Devillers, M. Paquet et al., “HIF1 activity in granulosa cells is required for FSH-regulated Vegfa expression and follicle survival in mice,” Biology of Reproduction, vol. 90, no. 6, article 135, 2014.

[20] R. C. Guzman, J. Yang, L. Rajkumar, G. Thordarson, X. Chen, and S. Nand, “Hormonal prevention of breast cancer: mimicking the protective effect of pregnancy,” Proceedings of the National Academy of Sciences of the United States of America, vol. 96, no. 5, pp. 2520–2525, 1999.

[21] I. H. Russo and J. Russo, “Mammary gland neoplasia in long-term rodent studies,” Environmental Health Perspectives, vol. 104, no. 9, pp. 938–967, 1996.

[22] K. Brit+ A. Ashworth, and M. Smalley, “Pregnancy and the risk of breast cancer,” Endocrine-Related Cancer, vol. 14, no. 4, pp. 907–933, 2007.

[23] K. K. Tsilidis, N. E. Allen, T. J. Key et al., “Oral contraceptive use and reproductive factors and risk of ovarian cancer in the European Prospective Investigation into Cancer and Nutrition,” British Journal of Cancer, vol. 105, no. 9, pp. 1436–1442, 2011.

[24] L. Titus-Ernstoff, K. Perez, D. W. Cramer, B. L. Harlow, J. A. Baron, and E. R. Greenberg, “Menstrual and reproductive factors in relation to ovarian cancer risk,” British Journal of Cancer, vol. 84, no. 5, pp. 714–721, 2001.

[25] N. Gleicher, “Why are reproductive cancers more common in nulliparous women?” Reproductive BioMedicine Online, vol. 26, no. 5, pp. 416–419, 2013.

[26] F. Meier-Abt and M. Bentires-Alj, “How pregnancy at early age protects against breast cancer,” Trends in Molecular Medicine, vol. 20, no. 3, pp. 143–153, 2014.

[27] S. I. Byers, M. V. Wiles, S. L. Dunn, R. A. Taft, and S. R. Singh, “Mouse Estrous Cycle Identification Tool and Images,” PLoS ONE, vol. 7, no. 4, p. e35538, 2012.

[28] H.-J. Park, S.-J. Park, D.-B. Koo et al., “Progesterone production is affected by unfolded protein response (UPR) signaling during the luteal phase in mice,” Life Sciences, vol. 113, no. 1-2, pp. 60–67, 2014.

[29] S.-Y. Shin, H.-J. Lee, D.-S. Ko, H.-C. Lee, and W. I. Park, “The regulators of VEGF expression in mouse ovaries,” Yonsei Medical Journal, vol. 46, no. 5, pp. 679–686, 2005.

[30] A. M. Rosales-Torres, I. Alonso, M. Vergara et al., “Vascular endothelial growth factor isoforms 120, 164 and 205 are reduced with atresia in ovarian follicles of sheep,” Animal Reproduction Science, vol. 122, no. 1-2, pp. 111–117, 2010.

[31] R. Iorio, A. Castellucci, G. Rossi et al., “Mancozeb affects mitochondrial activity, redox status and ATP production in mouse granulosa cells,” Toxicology in Vitro, vol. 30, no. 1, pp. 438–445, 2015.

[32] B. Berisha, D. Schams, M. Kosmann, W. Amselgruber, and R. Einspanier, “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, vol. 65, no. 4, pp. 1106–1114, 2000.

[33] R. L. Stouffer, “The function and regulation of cell populations comprising the corpus luteum during the ovarian cycle,” in The Ovary, P. C. K. Leung and E. Y. Adashi, Eds., pp. 169–184, Academic Press, Waltham, Mass, USA, 2nd edition, 2004.

[34] G. W. Fearnley, G. A. Smith, I. Abdul-Zani et al., “VEGF-A isoforms program differential VEGF2 signal transduction, trafficking and proteolysis,” Biology Open, vol. 5, no. 5, pp. 571–583, 2016.

[35] S. Kilic, N. Tasdemir, N. Lortlar, B. Yuksel, G. Budak, and S. Batioglu, “Vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) immunoreactivities in rat ovaries and uterine tubes after tubal ligation: A controlled immunohistochemical study,” The European Journal of Contraception and Reproductive Health Care, vol. 13, no. 4, pp. 431–437, 2008.

[36] R. C. Zimmermann, E. Xiao, N. Husami et al., “Short-term administration of antivascular endothelial growth factor antibody in the late follicular phase delays follicular development in the rhesus monkey,” The Journal of Clinical Endocrinology & Metabolism, vol. 86, no. 2, pp. 768–772, 2001.

[37] T. H. Wang, S. G. Horng, C. L. Chang, and et al., “Human chorionic gonadotropin-induced ovarian hyperstimulation syndrome is associated with up-regulation of vascular endothelial growth factor,” The Journal of Clinical Endocrinology & Metabolism, vol. 87, no. 7, pp. 3300–3308, 2002.

[38] M. Simons, E. Gordon, and L. Claesson-Welsh, “Mechanisms and regulation of endothelial VEGF receptor signalling,” Nature Reviews Molecular Cell Biology, vol. 17, no. 10, pp. 611–625, 2016.

[39] A.-K. Olsson, A. Dimberg, J. Kreuger, and L. Claesson-Welsh, “Mechanisms and regulation of endothelial VEGF receptors signalling—in control of vascular function,” Nature Reviews Molecular Cell Biology, vol. 7, no. 5, pp. 359–371, 2006.

[40] D. Klasa-Mazurkiewicz, M. Jarzab, T. Milczek, B. Lipińska, and J. Emerich, “Clinical significance of VEGFR-2 and VEGFR-3 expression in ovarian cancer patients,” Polish Journal of Pathology, vol. 62, no. 1, pp. 31–40, 2011.

[41] M. J. Waldner, S. Wirtz, A. Jefremow et al., “VEGF receptor signalling links inflammation and tumorigenesis in colitis-associated cancer,” The Journal of Experimental Medicine, vol. 207, no. 13, pp. 2855–2868, 2010.