VEGF in Non-Murine Animal Models of Normal Physiological Processes and Disease

Angiogenesis is a dynamic process that results in the formation of new blood vessels from pre-existing vasculature. This process is regulated by a host of pro- and anti-angiogenic factors (Iruela-Arispe 1997). When there is an imbalance favoring pro-angiogenic stimuli, the angiogenic switch is thrown (Bergers 2003). Blood vessels dilate and become leaky, pericytes that surround and support mature blood vessels loosen their connections, and the vasculature basement membrane and extracellular matrix is degraded by proteases allowing endothelial cells to proliferate and migrate towards the angiogenic stimuli. As endothelial cells migrate through the angiogenic gradient, they adhere together in a tube structure and a lumen is formed. Pericytes then migrate and stabilize the new vessel. One of the most potent stimulators of blood vessel growth is vascular endothelial growth factor (VEGF) (Ferrara 1997).

VEGF is a 42 kDa glycoprotein homodimer that is found in six isoforms created by alternative mRNA splicing; $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$ (Robinson 2001). $VEGF_{165}$ is secreted and is the most biologically active toward endothelial cells (Ferrara 1997). The VEGF isoforms (VEGF-A) described above, fall into a family that is characterized by a cystine-knot motif and includes VEGF-A (VEGF), VEGF-B (found in adult myocardium, skeletal muscle, and pancreas), VEGF-C (involved in lymphangiogenesis), VEGF-D (found in heart, lungs, skeletal muscle, colon, and small intestine), and placenta growth factor (PlGF) (Robinson 2001, Hoeben 2004).

Tyrosine kinase receptors for VEGF (fms-like tyrosine kinase (Flt-1) or VEGFR-1, kinase domain region (KDR) or VEGFR-2) are found exclusively on endothelial cells (Hoeben 2004). VEGFR-3 (Flt-4) functions in lymphangiogenesis. VEGF binding to VEGFR-2 induces endothelial cell migration and proliferation. Although VEGFR-1 has a higher affinity for VEGF than VEGFR-2, it has a weaker kinase activity upon VEGF binding and does not engender endothelial cell proliferation. Therefore, VEGFR-1 appears to be a decoy receptor and results in reduced VEGF-induced activity. The co-receptors, Neuropilins 1 and 2, enhance VEGF signaling. It is the balance between pro and anti-angiogenic factors and receptors that regulate vessel growth. Here, we will briefly highlight the role of VEGF in non-murine models of disease, normal physiologic processes, and tissue bioengineering.

When one thinks of angiogenesis, tumor growth often comes to mind. The volume of a tumor is limited to 1 mm^3, unless nutrients are brought in by a vascular supply (Folkman 1971). Hypoxic conditions in the tumor microenvironment up-regulate VEGF to stimulate vascular formation toward the tumor (Senger 1993). Circulating VEGF, along with VEGF in the tumor, has been used as a prognostic indicator for human tumor metastasis and patient survival (Ranieri 2006). VEGF has also been used for tumor prognosis in canines and felines. In one study, canines suffering from mammary gland tumors had significantly higher VEGF levels in their blood plasma and serum than canines with benign tumors (Kato 2007). Those canines that had high VEGF levels were significantly more likely to suffer from metastasis to the lungs (Kato 2007). Similarly, in a study of felines with invasive mammary carcinoma, the felines that had high tumor expression of VEGF had significantly reduced survival compared to felines with low VEGF expression in their tumors (Millanta 2002). Increased VEGF protein levels are not limited to solid tumors. In canine leukemia, acute leukemia (AL) and chronic lymphocytic leukemia (CLL), VEGF concentrations in the blood were at higher levels when canines presented with CLL compared with AL (Arico 2013a). Furthermore, the higher VEGF levels in CLL were correlated with a higher expression of matrix metaloprotease-9 (MMP-9) expression. MMP-9 cleaves extracellular matrix components, allowing endothelial cell migration. The same laboratory then assessed VEGF mRNA expression in canine lymphoma and found a statistically significant increase in VEGF mRNA in T-cell lymphoma compared to normal lymph nodes (Arico 2013b). There was also an increase in VEGF expression in

high grade T-cell lymphoma as opposed to low-grade T-cell lymphoma. The increased VEGF mRNA expression, once again, paralleled increased matrix metaloprotease-9 (MMP-9) expression in this cancer.

Tumor growth and metastasis is not the only pathological process that harbors an angiogenic imbalance. Angiogenesis in the eye, post development, results in a host of ocular diseases, including age-related macular degeneration (AMD) and diabetic retinopathy (DR) (Campochiaro 2013). In the wet form of AMD, choroidal blood vessels breach the Bruch's membrane and extend into the subretinal space. This results in vessel leakage, an accumulation of blood or serum beneath the retinal pigment epithelium, and subsequent vision loss. VEGF is a major stimulator of choroidal blood vessel neovascularization (CNV) (Julien 2008). Animal models that portray most of the characteristics of human AMD are lacking. Eye models in the rabbit, however, appear promising in recapitulating CNV. In a study focused on developing a model for CNV, the eyes of adult Chinchilla Bastard rabbits were injected with adenovirus vectors encoding VEGF, or control, into the subretinal space (Julien 2008). VEGF was stable and detectable in the eyes for up to four weeks, and, aberrant vasculature formed in the subretinal space. The characteristics of CNV in this rabbit model were similar to those found in human CNV, establishing this as a reliable animal model for studying AMD.

Diabetic retinopathy is another major cause of blindness in adults. This ocular disease is characterized by blood vessel blockage, followed by the growth of new, leaky blood vessels to compensate for the loss of oxygen and nutrient transport. In particular, DR has been linked to increased expression of the transcription factor hypoxia-inducible factor-1 (HIF-1), which, up-regulates VEGF (Campochiaro 2013). Treatments for inhibiting VEGF have been the norm for treating DR. In one animal model of DR, the eyes from New Zealand White rabbits were injected with adenoviral vectors containing VEGF165 (Viita 2009). This resulted in a significant increase in the size and number of capillaries in the eye, which resembled human DR neovascularization. The injection of adenovirus carrying 15-lipoxygenase-1 (15-LO-1), an oxidizing enzyme that has both pro- and anti-tumorigenic characteristics, inhibited VEGF-induced neovascularization. This occurred by inhibiting VEGF mRNA and protein expression. Current treatment options for these ocular diseases include antagonists to VEGF, such as ranibizumab, an antibody fragment that binds all isoforms of VEGF, and aflibercept, a recombinant protein that is constructed of VEGFR-1 and VEGFR-2 binding domains (Campochiaro 2013).

Angiogenesis at the wrong time of development causes disease, however, there are instances where angiogenesis is essential for normal physiological processes. These include the female reproductive cycle and wound healing. Angiogenesis takes on a cyclic function in the ovaries (Fraser 2006). Primordial follicles, containing immature oocytes, develop into pre-ovulatory follicles that enter the menstrual cycle. Once the egg has been ejected, the former follicle forms into a corpus luteum that supplies the hormone progesterone to support pregnancy. The maturation of these follicles, and the development and function of the corpus luteum, is dependent on angiogenesis (Fraser 2006, Fraser 2001).

A role for VEGF in follicular development in the ovary has been studied in bovines. An in vitro assessment of bovine fetal ovarian cortex exposed to various doses of VEGF revealed that VEGF was responsible for the transition of follicles from an immature to a more mature state (Yang 2007). As follicles mature, a dominant follicle arises that carries the egg to ovulation. A second bovine study suggested that VEGF (along with estradiol, progesterone, and insulin-like growth factor binding protein) could be used as a marker for dominant follicles, as dominant follicles contain significantly higher levels of VEGF than non-dominant follicles (Grazul-Bilska 2007).

VEGF is also involved in the vascularization of the corpus luteum during the estrous cycle and pregnancy. VEGF mRNA was detected in the newly formed corpus luteum, its level was reduced during corpus luteum regression, and VEGF mRNA levels increased, again, during pregnancy (Berisha 2000). The administration of a monoclonal antibody to VEGF during early corpus luteum development in Marmosets (primates) resulted in a 70% reduction in endothelial cell proliferation and a 60% reduction in the secretion of progesterone by the corpus luteum (Fraser 2001).

Angiogenesis, and VEGF, are critical for early fetal development during pregnancy (Andraweera 2012). Fetal growth reaches its peak in the 2nd half of gestation in humans and in other mammals. To meet the metabolic needs of the developing fetus, placental function and uterine blood flow must increase to support fetal growth (Reynolds 2005). Between 25 days and 32 weeks post conception, branching angiogenesis in the placenta is at its peak and VEGF is highly expressed (Kaufmann 2004). In maternal sheep placenta, the receptors for mRNA VEGF begin to increase between 14 and 16 days of pregnancy (Grazul-Bilska 2010) and VEGF mRNA more than doubles between 16 and 30 days of pregnancy (Grazul-Bilska 2011). VEGF mRNA peaked 130 days after the beginning of pregnancy in sheep maternal placenta and remained high in sheep fetal placenta between days 90 and 130 (Borowicz 2007).

VEGF is also essential for implantation of the embryo into the endometrium of the uterus. Uterine fluid was taken from women during the four-day window of time during the menstrual cycle that allows blastocyst implantation. Uterine fluid from women with infertility issues contained significantly less VEGF than those women with no fertility issues (Hannan 2011). Porcine studies confirm an increasing VEGF level in the uterus during pregnancy. VEGF mRNA levels in the uteran endometrium of the pig at Day 12 post conception were significantly increased over Day 1 levels (Wollenhaupt 2004). This was accompanied by an increase in both VEGFR-1 and VEGFR-2 mRNA levels. Additional studies reveal a role for progesterone in supporting VEGF expression in the uterus. In ovarectomzied pigs, the addition of progesterone increased VEGF mRNA levels in the endometrium (Welter 2003).

Pre-eclampsia, a complication during pregnancy, is characterized by high maternal blood pressure and kidney damage. Free (non matrix-bound) VEGF is reduced in the placenta in women suffering from pre-eclampsia, most likely due to an increase in the soluble form of VEGFR-1 (Andraweera 2012). Uteroplacental blood flow is also reduced in women suffering with pre-eclampsia. The importance of an adequate placental blood supply is supported by a study of Chinese Meishan and Yorkshire pigs (Vonnahme 2004). Meishan pigs have a similar uterine size, a similar ovulation rate, but a smaller placenta compared with Yorkshire pigs. However, Meishan pigs are capable of producing three to five more piglets per litter. The placental VEGF levels and placental weights in Meishan and Yorkshire pigs were observed from Days 30 to 110 post-conception. Meishan pigs were found to have a significantly better placental efficiency (fetal weight/placental weight) at Day 90 post conception. At Day 90, VEGF mRNA was also higher in the Meishan pig placentas. This parallels the increased vascularity found in Meishan placentas on Days 70 through 110 compared with their Yorkshire counterparts. Thus, a robust angiogenic response in the placenta could account for increased litter sizes found in Meishan pigs, and supports the requirement of sufficient levels of placental VEGF for a healthy pregnancy.

In addition to the female menstrual cycle and pregnancy, angiogenesis and VEGF are also necessary components in another physiological process: wound healing. Wound healing consists of four steps:

coagulation, inflammation, proliferation, and remodeling. Angiogenesis occurs during the proliferation phase, as nutrients, gas, and metabolites in the blood are required for the migration and proliferation of dermal and epidermal cells into the wound bed (Demidova-Rice 2012). Due to the similarity in structure between human and porcine dermis and epidermis, wound repair models in the pig have helped advance treatment options for chronic wound repair (Koyama 2011).

Age is one factor that plays a role in wound repair. Two-month-old, 4-month-old, and 24-month-old pigs were surgically wounded and followed for wound re-epithelialization and wound serum VEGF levels over 11 days (Yao 2001). The wounds from the older age group healed more slowly and had lower levels of VEGF in the wound serum than the younger age groups. After 11 days, an average of 79% of the area of the wounds on 2-month-old pigs were re-epithelialized, 48% on 4-month-old pigs, and 22% on 24-month-old pigs. By Day 4 after wounding, VEGF levels in the wound serum peaked in 2- and 4-month-old pigs (482 pg/ml and 419 pg/ml, respectively), however, it was only on Day 6 after wounding that the wound serum levels of VEGF peaked in the 24-month-old pigs at 229 pg/ml. This study correlates increased age with a lower production of VEGF, and thus, a slower wound healing process.

Preclinical wound-healing models involve extra cellular matrix scaffolds and tissue-engineered dermis to attract blood vessels to the wound bed. One such model uses collagen gels to keep the wound moist, supply structure for migrating and proliferating cells, and elicit cell signaling (Elgharably 2013). Wounds made in pigs that received collagen gel dressings resulted in significantly higher VEGF gene expression than control wounds 7 days post-wounding. On Day 21 post-wounding, wound beds also contained significantly more endothelial cells than control wounds, indicating an increased vasculature supply. A second model uses adenoviral VEGF transfected into porcine skin particles, which are subsequently transplanted into wounds on the pig. Wounds that received VEGF-transfected skin particles had a statistically significant increase in blood vessel growth compared with wounds receiving control skin particles (Koyama 2011). In a third study, human umbilical chord mesenchymal stromal cells expressing VEGF were seeded onto a collagen-containing dermal matrix and transplanted into skin wounds on pigs (Han 2014). Within one week of transplantation, the wounds were positive for VEGF and had a high degree of vascularization. Within three weeks, the wound had completed re-epithelialization.

Tissue bioengineering, and the necessity of inducing blood vessel growth to the tissue, is not limited to wound repair. Whether it is engineering a vascular supply for chronic wounds, a diseased heart, or repair of bone, VEGF and other growth factors (fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF)) play critical roles in establishing a viable blood network (Serbo 2013). In order to engineer new tissue, cells and growth factors are loaded onto a 3D scaffold before injection into a human or animal. Extracellular matrix-derived scaffolds (usually collagen-based) and biodegradable scaffolds are two types of platforms that are loaded with cells and growth factors in preclinical animal studies (Serbo 2013). VEGF may be incorporated into scaffolds in an encapsulated form or by coating scaffolds with heparin. (The negatively charged sulfate groups in heparin attract and bind the positive amino acids in VEGF). Localized and retained VEGF promoted blood vessel growth into the scaffold (Serbo 2013). The release of growth factors that promote angiogenesis can be temporally regulated. For instance, VEGF, FGF, and angiopoietin-2 (Ang-2) can be engineered to be released first, as these factors disrupt pre-existing vasculature and promote the migration of endothelial cells and pericytes to from new vasculature (Lee 2010). This may be followed by the release of angiopoietin-1 and

platelet-derived growth factor to stabilize the newly formed vessels (Lee 2010). The potential medical uses for bioengineered VEGF-stimulated tissue growth are extensive and are becoming a reality.

In summary, VEGF is a robust angiogenic stimulator that is tightly controlled to support normal physiological processes, such as wound healing, and the female reproductive cycle and pregnancy. As one of the main regulators of angiogenesis, an imbalance of VEGF can cause pathological responses, including tumor growth and metastasis, diabetic retinopathy, and age-related macular degeneration, to name only a few. Non-murine animal models are important in angiogenesis research, as these additional animal models have characteristics that mimic human disease states and physiological processes.

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