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# Vascular endothelial growth factor restores delayed tumor progression in tumors depleted of macrophages

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ABSTRACT

Genetic depletion of macrophages in Polyoma Middle T oncoprotein (PyMT)-induced mammary tumors in mice delayed the angiogenic switch and the progression to malignancy. To determine whether vascular endothelial growth factor A (VEGF-A) produced by tumor-associated macrophages regulated the onset of the angiogenic switch, a genetic approach was used to restore expression of VEGF-A into tumors at the benign stages. This stimulated formation of a high-density vessel network and in macrophage-depleted mice, was followed by accelerated tumor progression. The expression of VEGF-A led to a massive infiltration into the tumor of leukocytes that were mostly macrophages. This study suggests that macrophage-produced VEGF regulates malignant progression through stimulating tumor angiogenesis, leukocytic infiltration and tumor cell invasion.



## 1. Introduction

The development of a vascular network in tumors is a crucial step for the transition of the tumor to malignancy and is thought to be rate-limiting. However, the mechanism regulating this process, known as the angiogenic switch, is still largely unknown. Several angiogenic factors including acidic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), transforming growth factor-α (TGF-α), TGF-β, hepatocyte growth factor, and tumor necrosis factor-α (TNF-α) have all been identified to play an important role in regulating tumor angiogenesis. Among these factors, VEGF-A is

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a potent angiogenesis promoter for blood vessel growth in both physiological and pathological conditions (Ferrara, 2004). It regulates the survival and proliferation of endothelial cells as well as being a potent inducer of vascular permeability (Dvorak, 2002; Ferrara et al., 2003).

VEGF-A is a member of a gene family that includes placenta growth factor (PIGF), VEGF-B, VEGF-C and VEGF-D (Ferrara et al., 2003; Ferrara, 2004). In the mouse, alternative splicing leads the generation of four protein isoforms of VEGF-A (Shima et al., 1996) and among them, $VEGF_{164}$ is the predominant molecular species found in variety of normal and transformed cells (Keyt et al., 1996). The activity of VEGF-A is mediated by VEGF-R1 or FMS-like tyrosine kinase 1 (Flt-1) and VEGF-R2 (Flk-1), both of which are expressed by endothelial cells (Ferrara, 2004). VEGF-A is an endothelial mitogen (Leung et al., 1989). In addition to promoting the development of blood vessels, VEGF-A has also been reported as a chemoattractant for endothelial cells, osteoclasts and monocytes (Yoshida et al., 1996), the latter of which express VEGF-R1.

The expression of VEGF-A has been found in various human cancers and it is associated with disease progression and decreased survival rate, suggesting that it plays an important role in malignant progression (Jain et al., 2006). In addition to tumor cells, VEGF is also expressed in the tumor stroma, raising the question of the relative importance of VEGF produced in each compartment in tumor angiogenesis and progression. Dong et al. (2004) reported that VEGF-null fibrosarcomas were tumorigenic and angiogenic *in vivo* in spite of the absence of tumor-derived VEGF. This group further demonstrated that VEGF-producing stromal fibroblasts played a crucial role in regulating tumor angiogenesis in the transplanted VEGF-null fibrosarcomas. Using a mouse model of cervical cancer, Giraudo and coworkers also reported that macrophages in the tumor microenvironment regulating tumor angiogenic switch by producing MMP-9, a protease implicated in mobilization of VEGF (Giraudo et al., 2004). These studies indicate that in certain tumor microenvironments, stromal cells play a dominant role in promoting the angiogenic switch through producing VEGF or affecting the function of VEGF.

We have previously shown that VEGF-A is produced by tumor-associated macrophages in a mouse model of breast cancer, PyMT mice (Lin et al., 2006). Depletion of macrophage growth factor, CSF-1, which reduced the infiltration of macrophages into the tumor stroma, resulted in a delayed tumor angiogenic switch and malignant transition in this model (Lin et al., 2001, 2006). These observations suggest that in the mammary tumors of PyMT mice, macrophage-produced VEGF-A regulates the growth and development of blood vessels during the angiogenic switch. The fact that CSF-1 is able to stimulate the expression of VEGF-A in cultured macrophages also supports this notion (Eubank et al., 2003). Based on these studies, we hypothesize that VEGF-A produced by CSF-1 dependent-macrophages is required for the onset of the angiogenic switch in PyMT model of breast cancer. To test this hypothesis, we established a strain of transgenic mouse in which the expression of VEGF-A was expressed in the mammary epithelium in a temporal specific manner. We demonstrate that restoring VEGF-A expression in the premalignant mammary lesions of CSF-1 depleted mice promotes their tumor progression to the level of their wild-type counterparts. Therefore, the lack of macrophage-produced VEGF-A is a key element contributing to the delayed tumor progression to malignancy in CSF-1 depleted PyMT tumors and can be compensated for by tumor produced VEGF-A.

## 2. Results

### 2.1. *Regulated expression of VEGF in the mammary gland*

To achieve conditional expression of VEGF-A specifically in the mammary epithelium, we have established a bi-transgenic mouse line using the tetracycline-regulated repressor-transactivator system. In this system, the expression of the transgene, VEGF-A-encoding cDNA, is under the control of a tetracycline-dependent promoter (Figure 1A). The activity of this promoter is regulated by the reverse tetracycline-dependent transcriptional activator, rtTA, whose expression is under the control of mouse mammary tumor virus (MMTV) promoter, as reported previously, that shows specific expression in the mammary epithelium and to a lesser degree the salivary gland but not elsewhere (Mok et al., 1992). In the bi-transgenic mice, transcription from the transgenic VEGF is induced in the presence of doxycycline (Dox). To monitor the expression of the transgene *in vivo*, a foot-and mouth disease virus (FMDV) 2A DNA segment (Donnelly et al., 2001) was added in-frame at the 3′ of the VEGF-A and 5′ of GFP cDNAs as illustrated in Figure 1A. This construct permits multi-cistronic expression of VEGF-A, 2A and GFP mRNA. However, as the 2A sequence directs cleavage of the polypeptide during translation (de Felipe, 2004), VEGF-A and GFP are synthesized in the same cell but as two separate proteins. This approach marks sites of expression while at the same time preventing the potential aberrant function of a GFP–VEGF-A fusion protein. Furthermore, to reduce leaky transcription from the transgene, a common problem of the tetracycline regulatory system, a silencer construct was co-injected with the transgene (data not shown). No leakage was found in untreated transgenic mice (data not shown).

One founder carrying the *Vegfa-2A-Gfp* transgene (*Tg(TetopVegfa-2A-Gfp)1Jwp*) that had co-integrated with the Tet-silencer was identified (referred as VEGF-A bi-transgenic) and bred onto mice carrying the MMTV LTR/rtTA transgene (*Tg(MMTV-rtTA)Lach*). The expression of VEGF-A and GFP in the mammary glands of the bi-transgenic mice was compared with mice carrying one of the transgenes using Western blotting analysis (Figure 1B). Both the transgenic and control mice were treated with 1 mg/ml doxycycline (Dox) in drinking water for 2 weeks from age 6 to 8 weeks. The levels of both VEGF-A and GFP protein were strongly induced in the mammary glands of the bi-transgenic mice (Figure 1B, lanes 1 and 2) compared to undetectable signals in mice carrying either one of the transgenes (Figure 1B, lanes 3–5).

To confirm that the expression of VEGF-A is restricted to the mammary gland, different tissues from bi-transgenic mice with the same treatment were analyzed. Figure 1C