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Impact of VEGF Expression on the Physiological Characteristics of Clonal Cell Lines

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Abstract. *Background: Vascular endothelial growth factor (VEGF) plays a crucial role in tumor angiogenesis and growth in most solid neoplasia. Materials and Methods: Clonal tumor cell lines expressing varying levels of this pro-angiogenic factor were created via recombinant adeno-associated virus infection of a human (HT29) and rodent (SCCVII) tumor model. Results: The alteration in VEGF expression levels did not significantly impact the in vitro growth rate of the clonal cell lines or the expression levels of other known pro-angiogenic factors. However, the tumors that arose from these clonal cell lines did display significant physiological differences. Up-regulation of VEGF expression increased the in vivo growth rate and the intratumoral vessel density of the resulting tumors and decreased the extent of tumor necrosis. Conclusion: Since the tumor vascular network can impact the efficacy of anti-cancer therapies, these results suggest that VEGF expression may be important to consider in the treatment of cancer.*

Angiogenesis, the growth of new blood vessels from existing vasculature, is an important physiological process that is fundamental in wound healing, reproduction and development (1). Endothelial cells are usually quiescent in normal tissues, dividing, approximately, once every seven years. However, in a variety of pathological disorders, including cancer, the growth rate of endothelial cells can be rapidly accelerated, with divisions occurring as fast as once a week (2). This "angiogenic switch" has been shown to be vital to the growth of a tumor beyond a diameter of, approximately, 1-2 mm (3).

The complex process of angiogenesis is a tightly regulated balance between endogenous factors that can either promote or inhibit new vessel growth (4). Angiogenic stimulators include basic fibroblast growth factor (bFGF) (5), platelet-derived growth factor (PDGF) (6), the angiopoietins (Ang-1 and Ang-2) (7, 8) and the vascular endothelial growth factor (VEGF) (9). Of these, VEGF is considered to be the key regulator of growth in a wide variety of tumor types (10).

The role of VEGF in clinical studies has not been so clear. Some studies have suggested that a high pre-treatment level of serum and/or tumor VEGF expression is a prognostic indicator of poor survival (11-13). Other studies have shown the opposite relationship (14) or no relationship at all (15). A number of factors confound the inter comparison of data collected across clinical studies including differences in the genetic background of tumors, end-points assessed, and sample collection techniques.

Contradictory results have also been seen when the issue of tumor vascularity and its impact on treatment outcome was addressed. Several studies that examined the relationship between vessel density and radiation therapy have shown that decreased intratumoral vascularity leads to a poor outcome (16, 17), whereas others have shown that increased vascularity is associated with better tumor control (18). In addition, a large study involving head and neck tumors has shown a "U-shaped" response, where both increased and decreased intratumoral vascular density resulted in a poor outcome (19). However, it can be difficult to draw firm conclusions from these observations since there are several inter-study differences, including tumor types studied, vessel visualization techniques, and vessel density determination methods (*i.e.*, hot spot *versus* distance to closest vessel).

To avoid such complications in this study, clonal cell lines varying in VEGF expression were created from two tumor types: a human colon carcinoma (HT29) and a murine squamous cell carcinoma (SCCVII). In both cases, the parental cell line was infected with an adeno-associated virus (AAV) containing the gene for human VEGF and stable cell lines expressing three different levels of this pro-angiogenic gene were generated. These clonal cell lines, as well as the tumors that arose from them, were examined for their growth properties and physiological characteristics.

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Materials and Methods

Cell culture. Human HT29 colon carcinoma cells were grown in Dulbecco's modified minimum essential medium (DMEM) (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) 1% penicillin-streptomycin (Invitrogen) and 2 mmol/L L-glutamine (Invitrogen). Murine SCCVII squamous cell carcinoma cells were grown in alpha minimal essential media (α-MEM) supplemented with 10% FBS (Invitrogen) 1% penicillin-streptomycin (Invitrogen) and 2 mmol/L L-glutamine (Invitrogen).

Generation of stable cell lines by rAAV transduction. HT29 or SCCVII cells were infected with a recombinant adeno-associated virus (rAAV) containing the cassette for human $VEGF_{165}$, as previously described for the human endostatin gene (20). Briefly, $1x10^4$ HT29 or SCCVII cells were suspended in 50 μL of serum- and antibiotic-free medium. A rAAV containing the $VEGF_{165}$ gene and a neomycin resistance gene were then added at a multiplicity of infection (MOI) of 10,000, and the mixture was incubated for 3 h at 37˚C. Cells were then grown in selection media that contained 1 mg/mL geneticin for 48 h. Cells were plated at a low density in 60-mm dishes to obtain clones. Stable cell lines were maintained in the appropriate cell media with the addition of 500 μg/mL geneticin.

Angiogenesis factor expression. HT29 or SCCVII cells were plated in 60-mm dishes at a density of $2x10^6$ cells in 2 mL of cell culture media. Following a 24 h incubation period, the cell culture supernatants were collected and analyzed by ELISA (R&D Systems, Minneapolis, MN, USA) for the following factors: VEGF, PDGF, bFGF, Ang-1 and Ang-2.

In vitro cell growth. HT29 or SCCVII cells were plated in 60-mm dishes at a density of $1x10^4$ cells. At various times thereafter, cells were trypsinized and counted using a hemocytometer. The average number of cells per plate (3 plates per time point) was determined as a function of time after plating.

Animals and tumor models. Mice were injected with either $1x10^6$ HT29 tumor cells or $1x10^5$ SCCVII tumor cells intramuscularly [in a volume of 0.02 mL phosphate-buffered saline (PBS)] into a single hind limb of 6- to 8-weeks-old female NCR nu/nu or C3H/HeJ mice (Frederick Cancer Research Facility, MD, USA), respectively. The mice were maintained under specific pathogen-free conditions (University of Florida Health Science Center Vivarium, Gainesville, FL, USA) with food and water provided *ad libitum*.

In vivo growth rate. For both the HT29 and SCCVII models, tumor size was measured by passing the tumor bearing leg through a series of increasing diameter holes in an acrylic plate. The smallest diameter hole that the tumor-bearing leg could pass through was recorded and converted to a tumor volume using the following formula:

$$\text{tumor volume} = 1/6(\pi d^3)-100,$$

where d is the hole diameter and 100 represents a volume correction factor determined for a mouse leg without a tumor. The times for the tumors to grow from appearance (approximately 200 mm^3) to 1,000 mm^3 was recorded.

Immunohistochemistry of CD31. Frozen sections of tumors arising from each of the clonal cell lines were cut on a cryostat, air-dried, and fixed in acetone/methanol at 4˚C for 10 min. Tumor microvessels were stained using a mouse monoclonal antibody to the CD31 (PECAM-1) antigen found on endothelial cells (Beckman Coulter, Brea, CA, USA), applied overnight at 4˚C at a dilution of 1:50. A secondary antibody conjugated with Cy3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was applied for 1 h at room temperature. The staining was followed by standard washing and then slides were allowed to air-dry prior to storage at 4˚C.

Assessment of vascular density. Tumor sections stained for the CD31 antigen were viewed and captured using a Zeiss Axiophot 2 microscope (Carl Zeiss Jena GmbH, Jena, Germany) with a Sony DXC970 color camera (Sony Corporation, Tokyo, Japan). The entire tumor section was reconstructed by tiled field mapping. Estimates of tumor angiogenesis were made using the NIH ImageJ software with an automatic threshold setting to generate the percentage of tumor area that contained blood vessels.

Assessment of necrotic fraction. HT29 or SCCVII tumors were fixed in formalin, embedded in paraffin, sectioned, and H&E stained by the University of Florida molecular pathology core facility. Pictures of the entire tumor section were taken using a morphometric microscope and these pictures were analyzed using the software program ImageJ (NIH). The necrotic fraction was obtained by dividing the area of necrosis by the total tumor section area.

Intradermal angiogenesis assay. In situ tumor-induced angiogenesis was evaluated using an intradermal assay that has been previously described (20). Briefly, a solution of $1x10^5$ HT29 or SCCVII cells was injected intradermally in a volume of 10 μL of PBS at four sites on the ventral surface of a female nude mouse. Three days post-injection the mice were killed *via* cervical dislocation and the skin was separated from the underlying muscle. The number of vessels intersecting the tumor inoculates were counted with the aid of a dissection microscope (5x magnification). The resultant data points for each group were pooled for statistical analysis.

Statistical analysis. The student's *t*-test was used to compare *in vitro* results that were reported as the mean ± standard deviation. *In vivo* comparisons were made using the Wilcoxon rank sum test for non-parametric paired variables where the median was presented. All analyses were made using the Systat software package (Systat, Richmond, CA, USA). *P*-values of less than 0.05 were considered as significant.

Results

HT29 and SCCVII cells were infected with a rAAV containing the gene for human $VEGF_{165}$ and colonies were selected for their expression of human VEGF. Three clones were chosen for each tumor model: the first clone expresses VEGF at a level that is comparable to the parental cell line, the second clone expresses VEGF at an intermediate level, and the third clone expresses VEGF at a high level (Figure 1).

Since many growth factor pathways are interconnected, it was conceivable that manipulating VEGF expression might

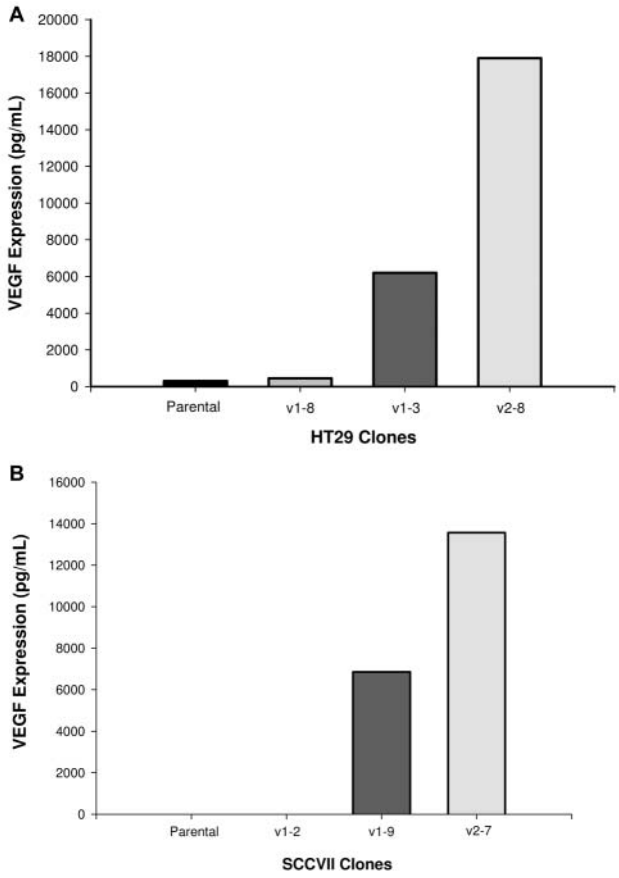

Figure 1. *Human VEGF expression levels of the HT29 (A) and SCCVII (B) clonal cell lines, as assayed by ELISA. Bars represent an average of two readings.*

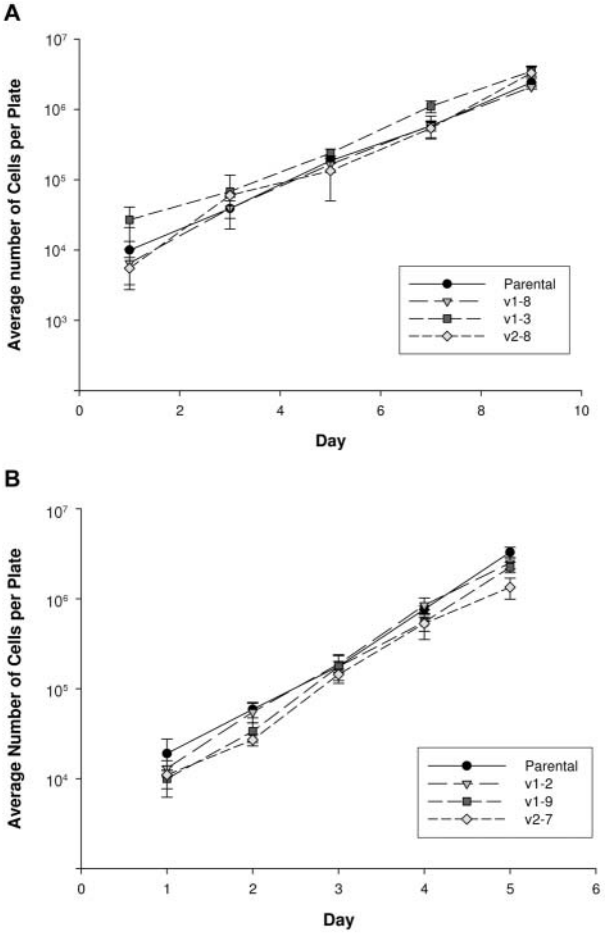

Figure 2. *Cell growth curve of the HT29 (A) and SCCVII (B) clonal cell lines as a function of time after plating. Symbols represent an average of 3 plates per time-point. No significant difference in growth rates was detected using the Student's t-test.*

also alter the expression of other pro-angiogenic factors. The clonal cell lines, therefore, were also tested for the expression levels of bFGF, Ang-1, Ang-2 and PDGF. Neither the parental HT29 or SCCVII nor the clonal cell lines derived from either model were found to have significant expression of these growth factors. In addition, RT-PCR was carried out to confirm the lack of expression of VEGFR1 and VEGFR2 on the tumor cell lines.

Studies assessing the inherent growth characteristics of the parental (non-infected) and clonal cell lines showed that the elevation of VEGF levels did not significantly alter the growth rates in either the HT29 or SCCVII tumor models (Figure 2). The *in vivo* time to appearance (~ 200 mm^3) and the subsequent growth rate (200 to 1,000 mm^3) of the tumors resulting from the parental and clonal cell lines were then determined. Figure 3A shows that the tumors arising from the mid- and high-level VEGF expressing clones of the HT29 cell line appear faster than the parental and low-level expressing clonal cell lines. Tumors resulting from the highest expressing VEGF clone also grew significantly faster from 200 to 1,000 mm^3 than the other groups (Figure 4A). This was not seen with the SCCVII tumors; there was no significant difference in the time to tumor appearance or the growth rate from 200 to 1,000 mm^3 among the different clones and the parental cell line (Figure 3B, 4B).

Since VEGF is known to be a potent pro-angiogenic factor, it was anticipated that vessel density and extent of tumor necrosis could vary among the clones and the parental cell lines. Indeed, tumors arising from the higher VEGF expressing clonal cell lines displayed significantly elevated vessel densities (Figure 5). To ascertain whether this was the consequence of greater angiogenic activity *in vivo*, small inoculates of tumor cells were injected intradermally on the ventral surface of mice and the number of vessels induced by the various clonal cell lines was determined. The results showed that the higher VEGF expressing clonal cell lines were able to induce a 2- to 3-fold

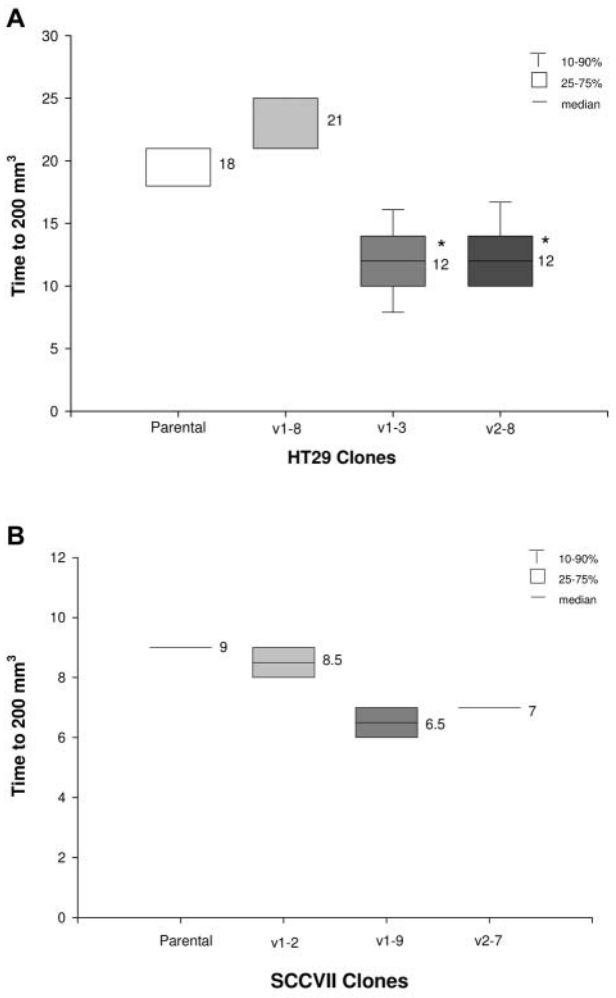

Figure 3. *1x10^6 HT29 tumor cells (A) or 1x10^5 SCCVII tumor cells (B) were injected i.m. into the left hind leg of nude mice. The number of days until the appearance of the tumor (~ 200 mm^3) was recorded. Asterisk (*) indicates a p-value of less than 0.05 when groups were compared via the Wilcoxon rank sum test to the parental group.*

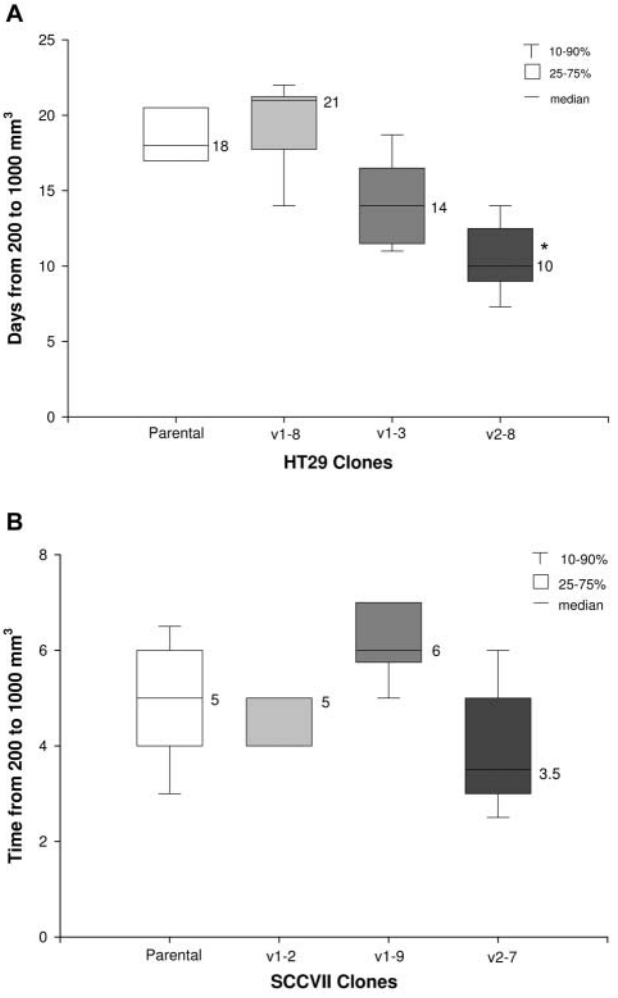

Figure 4. *1x10^6 HT29 tumor cells (A) or 1x10^5 SCCVII tumor cells (B) were injected i.m. into the left hind leg of nude mice. The number of days from the time of tumor appearance (~ 200 mm^3) until the tumor reached a size of 1000 mm^3 was recorded. Asterisk (*) indicates a p-value of less than 0.05 when groups were compared via the Wilcoxon rank sum test to the parental group.*

greater number of blood vessels to grow towards the tumor cell inoculates *in vivo* (Figure 6).

The increased vascularity observed in tumors derived from high VEGF expressing HT29 cells also led to a reduction in tumor necrosis from ~20% in control HT29 xenografts to <10% in tumors established from the v2-8 clone (Figure 7A). However, increasing vascularity did not affect the ~4 % necrotic fraction associated with SCCVII tumors (Figure 7B).

Discussion

VEGF is known to be a potent pro-angiogenic growth factor and the upregulation of its expression has been shown in a wide variety of tumors (21-23). Although some clinical studies have demonstrated the potential use of VEGF as a prognostic indicator (24, 25), not all studies agree with its use as a biomarker (26). This is perhaps not surprising given the wide range of factors that may influence the interpretation of clinical data.

In the present study, stable clonal cell lines that express different levels of VEGF while possessing the same genetic backgrounds were generated. Two tumor types, a human colon carcinoma (HT29) and a murine squamous carcinoma (SCCVII) origin, were used. Clonal cell lines were established from both parental types and were subsequently examined for their *in vitro,* as well as *in vivo* characteristics.

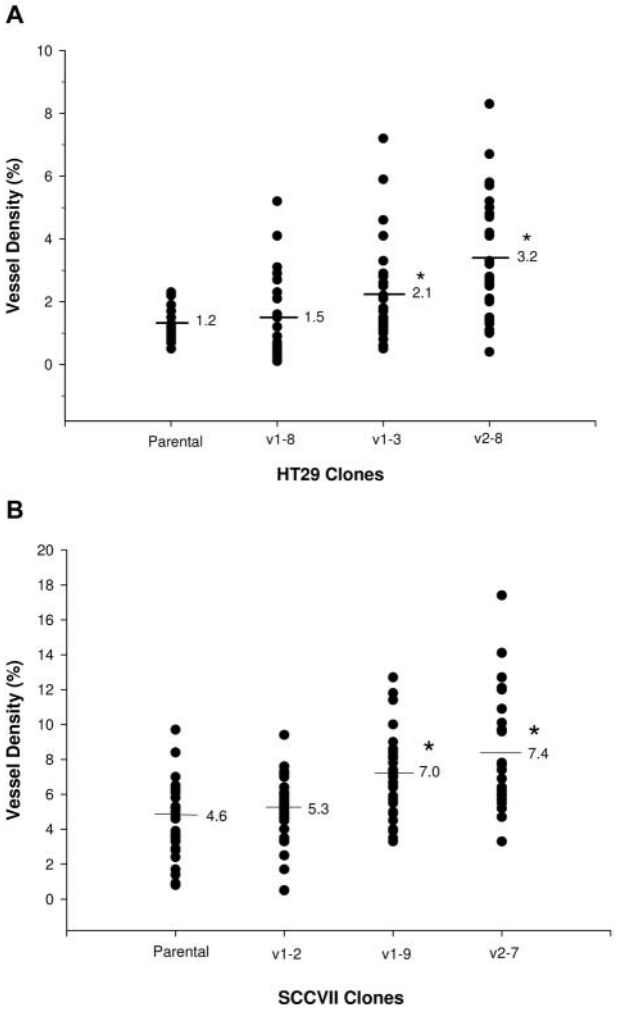

Figure 5. *HT29 tumors (A) and SCCVII tumors (B) resulting from the clonal and parental cell lines were stained for the CD31 antigen. Vessel density was obtained by dividing the vessel stained area by total area. Value indicated by a horizontal line is the median value of group data points. Asterisk (*) indicates a p-value of less than 0.05 when data points were compared to control data points as computed by the Wilcoxon rank sum test.*

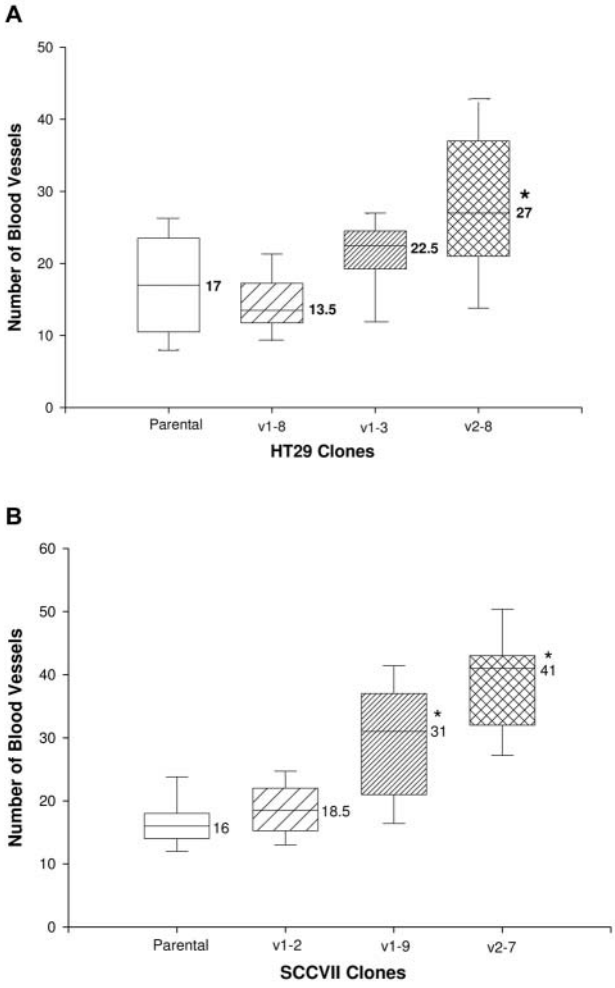

Figure 6. *1x10^5 HT29 tumor cells (A) or SCCVII cells (B) were injected at 4 sites on the ventral surface of a nude mice. Seventy-two h later the mice were killed and the skin flap containing the inoculation sites was excised. The number of blood vessels intersecting each inoculate was counted and data points for each group were pooled. Asterisk (*) indicates a p-value of less than 0.05 when groups were compared via the Wilcoxon rank sum test to the parental group.*

Manipulation of the VEGF expression levels did not significantly affect the *in vitro* growth rate or the expression levels of other pro-angiogenic growth factors: bFGF, PDGF, Ang-1 and Ang-2. However, the *in situ* characteristics of tumors arising from the various clones were affected. Tumors derived from the HT29 clonal cell lines clearly showed that an increase in VEGF expression resulted in an earlier tumor appearance, as well as an increased growth rate. This was not seen with the SCCVII clonal cell lines. One possible explanation for the lack of an effect of VEGF expression on SCCVII growth may be that the inherently high *in situ* growth rate of the parental line of this model minimizes the possibility of further enhancement in the growth rate.

The visualization of endothelial cells with the CD31 antigen demonstrated that an increase in the cellular expression levels of VEGF is related to an increase in the vessel density of the resulting tumor. This observation is not altogether surprising since VEGF is a potent angiogenic factor. Taken together, the higher level of intratumoral vessel density that was seen with the increased cellular VEGF expression may be the explanation for the increased *in vivo* growth rate that has already been noted. Indeed, other studies, that have used siRNA to target VEGF, have noted that reductions in VEGF results in a decrease in the intratumoral vessel density and marked suppression of *in vivo* tumor growth (27-29).

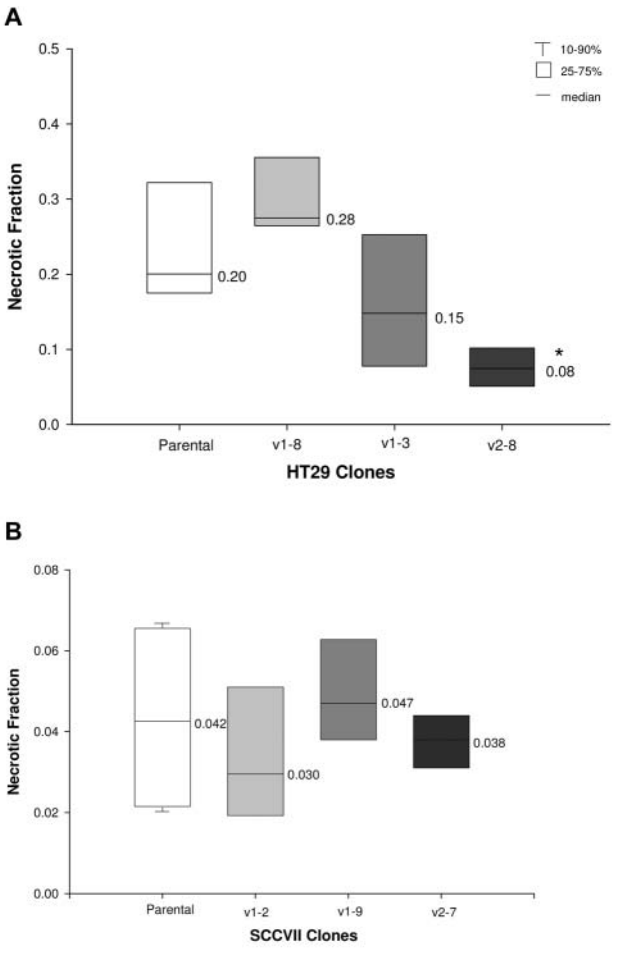

Figure 7. *Paraffin sections of the HT29 tumors (A) and SCCVII tumors (B) resulting from the clonal and parental cell lines were H&E stained. The necrotic fraction was obtained by dividing the area of necrosis by the total tumor section area. Asterisk (*) indicates a p-value of less than 0.05 were data points were compared to control data points via the Wilcoxon rank sum test.*

An increase in vessel density within a tumor should result in increased nutrient delivery and enhanced survival of tumor cells. As expected, the increased vascularity associated with tumors derived from increased VEGF expressing HT29 clones also decreased the extent of necrosis in the xenografts ~2-fold compared to parental tumors (Figure 7A). Such a reduction in necrosis was not seen in SCCVII tumors of varying vascularities, probably because the very low level of necrosis, present even in the parental tumors of this cell line (~4%), would make it difficult to demonstrate any significant change in necrotic fraction.

In conclusion, the results of this study show that clonal cell lines expressing various levels of the pro-angiogenic factor VEGF give rise to tumors with altered vascularity. Since tumor vascularity affects the distribution of nutrients, such as oxygen, as well as the delivery of chemotherapeutic agents, the inadequate and non-uniform vascular network within a growing tumor has been linked to the failure of many anti-cancer therapies (30). The genetic approaches used in the present study established tumor models which may be applied to directly examine the relationship between angiogenic factors, tumor vascularity and treatment outcome without the confounding difficulties typically associated with inter-tumor comparisons.

Acknowledgements

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