Noninvasive indirect imaging of vascular endothelial growth factor gene expression using bioluminescence imaging in living transgenic mice

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Vascular endothelial growth factor (VEGF) has been recognized as one of the main regulators of angiogenesis and plays an important role in neovascularization occurring under both physiological and pathological conditions (9, 32). Upregulation of VEGF production has also been demonstrated during wound repair in keratinocytes in skin wounds in rat and mice (3). Early stages of wound repair are characterized by increased vascular permeability, and the role of VEGF in wound healing became apparent with increased VEGF production in skin wounds (3). The role of VEGF in angiogenesis during wound healing has been well documented (3, 6, 24).

Vascular endothelial growth factor (VEGF) has been recognized as one of the main regulators of angiogenesis and plays an important role in neovascularization. Several tumor types are known to express potent angiogenic cytokines such as basic fibroblast growth factor (FGF-2), VEGF, and interleukin-8 (1, 19, 26). Because the progressive growth of tumors beyond a minimum size is dependent on angiogenesis, the early activation of adjacent stromal tissues with VEGF is a significant event in tumor cell proliferation and survival. VEGF is known to be upregulated in many cancers, inflammatory responses, and ischemic conditions (9, 30).

VEGF gene expression is also strongly controlled by environmental stress, such as hypoxia and hypoglycemia. VEGF levels are upregulated under hypoxic conditions and stimulate angiogenesis to support wound healing or tumor growth (21). VEGF expression has also been known to be upregulated in the presence of heavy metal ions (8). VEGF’s role as an angiogenesis initiator has led to its recognition as a potential target for antiangiogenic therapies. The ability to monitor upregulated VEGF levels in vivo in a noninvasive and quantitative manner will allow monitoring of early events during angiogenesis. The potential of noninvasive molecular imaging as a modality to monitor reporter gene expression in living animals has been realized in several applications (11, 12, 13). These include, among others, the use of positron emission tomography (PET) and bioluminescence imaging (BLI) (4, 14, 22, 33).

Presently, there are not many direct approaches to monitor endogenous VEGF levels during wound healing, although the indirect approaches described next have been attempted. Transgenic mice carrying VEGF promoter (pVEGF) have been used to study the expression of green fluorescent protein (gfp) reporter gene during wound healing and tumor growth (10). Transgenic mice carrying the human VEGF promoter were also used to detect VEGF promoter activity in the skin (18). These approaches have been somewhat limited due to poor depth penetration of light, autofluorescence leading to background signal, and lack of quantitation. The advent of recent techniques such as multichannel imaging technology and fluorescence-mediated optical tomography may help to overcome these issues and lead to their increased use in monitoring early VEGF activation (20, 25). We have been exploring ways to indirectly measure VEGF gene expression in living mice using in vivo BLI. In the recent past, BLI has been increasingly used for the real-time analysis of gene expression in small animal models (4, 5). Technological advancements have led to the development of highly sensitive detection cameras capable of imaging small levels of light emitted from rodent tissues. A major advantage of using BLI is the very low level of back-
ground signal compared with fluorescence-based imaging strategies. We have been studying the transcriptional activity of pVEGF using firefly luciferase (fl) in living mice. Our initial work in cell culture and in vivo using the vector representing the one-step system (pVEGF-fl) resulted in relatively low levels of fl expression (unpublished data). To enable sensitive monitoring of VEGF induction using in vivo imaging assays, the level of gene expression must be considerably higher. Therefore, strategies to augment the transcriptional activity of pVEGF were warranted. We have previously demonstrated that the transcriptional activity of the weak, prostate-specific promoter can be significantly enhanced using the yeast transcriptional activator GAL4-VP16 (17). This strategy, known as the two-step transcriptional amplification (TSTA) system, resulted in a 50-fold increase in prostate-specific fl expression over the one-step system (prostate-specific promoter directly driving fl expression). In the present study, we extended the utility of the TSTA system to augment pVEGF-driven fl expression levels.

The objectives of the present study were 1) to enhance the transcriptional activity of pVEGF and evaluate its induction efficacy in cell culture using hypoxia and cobalt chloride and 2) to create a transgenic mouse model to monitor induction of pVEGF-mediated fl expression during wound healing and tumor growth.

MATERIALS AND METHODS

Construction of plasmids. pVEGF-fl was made by cloning a 3,019-bp Kpn I to HindIII genomic fragment (−2,274 to +745) containing the human pVEGF (31) upstream of fl cDNA in a pGL2-basic vector (Promega) (23). pVEGFVP2.G5-fl was made by ligating the Kpn I/HindIII fragment of human pVEGF from pVEGF-fl with the HindIII/Not I fragment of GAL4VP2 and the Not I/Kpn I fragment of G5E4T-fplGL3 from the single TSTA vector pBCVP2.G5-fl (35). pVEGFVP2.G5-fl was made by ligating three DNA fragments, the Kpn I/HindIII fragment of human pVEGF, the HindIII/Not I fragment of GAL4VP2, from plasmid pSEBCGAL4VP2 (35), and the Not I/Kpn I fragment of G5E4T-fplGL3. pVEGFVP2.G5-fl was made by cloning the G2-fragment into Kpn I and Sal I sites of pVEGFVP2.G5-fl. Note that “p” refers to the plasmid, and “p” denotes the promoter. The number of VP16 activation domains in the construct was one and two (VP1 and VP2) (7). In the reporter template, the number of Gal4-binding sites upstream of fl varied from one to five (G1–fl, G2–fl, and G5–fl). The three single vectors generated by combining the effector and reporter templates are pVEFGGAL4VP2.G5–fl (pVEGFVP2.G5-fl), pVEFGGAL4VP2.G5–fl (pVEGFVP2.G5–fl), and pVEFGGAL4VP2.G5–fl (pVEGFVP2.G5–fl) (Fig. 1A). Figure 1B illustrates the construct used to generate transgenic mice. Note that GAL4 refers to the transactivator, and Gal4 refers to the binding sites placed upstream of the fl gene.

Cell lines and transfection conditions. HeLa (human cervical carcinoma) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). NK2 (FVB/N mouse mammary tumor) cells were a kind gift from Timothy Lane’s laboratory at the University of California, Los Angeles (UCLA). HeLa and NK2 cells were grown in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 0.2 mM glutamine, 100 units/ml penicillin-G, and 100 mg/ml streptomycin. The transfections were carried out using Superfect transfection reagent (Qiagen, Valencia, CA). Plasmids used in the transient transfection of HeLa cells are listed in Fig. 1A. Twenty-four hours after transient transfection, the cells were replated into nine plates with fresh medium and grown for 24 h. After 24 h, three of the nine plates were exposed to hypoxia for 48 h (1 or 0% O2, 5% CO2, 94% N2), three plates were incubated in the presence of cobalt chloride for 48 h (final concentration of 100 μM), and three were left under normal incubation conditions for 48 h. Stable clones (expressing pVEGF-fl and pVEGFVP2.G5–fl) were selected from HeLa cells with 500 μg/ml G418 and thereafter maintained at a concentration of 300 μg/ml. The stable cells were assayed for FL activity under hypoxia and cobalt chloride conditions at 6, 24, 32, 48, and 56 h.

Assay for FL activity. After 48-h induction, the transiently transfected cells were harvested and lysed with passive lysis buffer (Promega, Madison, WI). FL activity was measured using the luciferase assay kit from Promega in a luminometer (Turner Designs, Sunnyvale, CA). The relative light units were normalized to the micrograms of protein in each well. Triplicate observations were made at each time point.

Assay of VEGF protein by ELISA. Cell supernatants from triplicate plates were assayed for human VEGF165 isofrom using a human VEGF ELISA kit (Oncogene Research Products, Boston, MA). Color development was recorded on a 96-well plate reader (TECAN, Salzburg, Austria). The results were quantified using a linear range standard curve obtained with recombinant human VEGF as control.

Generation of pVEGF-TSTA-fl transgenic mice. To prepare a linear transgene for microinjection, the single TSTA vector in pGL3 backbone was digested with Not I and Sal I. The 1.2-kb pVEGF-VP2.G5–fl DNA fragment was purified by agarose gel electrophoresis and prepared for injection. The fragment was microinjected into fertilized embryos of FVB/N mice. The presence of fl in the transgenic founders was confirmed by PCR amplification of DNA obtained from tail biopsies using oligonucleotide primers 5′-CCGACTCTAGAGGATCCCGGCG-3′ (E4T) and 5′-CAGCGGATAGAATGGCGCCGGG-3′ (fl). About 0.5–1 cm of tail was digested in tail lysis buffer containing proteinase K (0.55 mg/ml). The DNA was precipitated with isopropanol, washed with ethanol, and resuspended in 50 μl of buffer. One microliter was used for PCR genotyping. The founders were crossed with wild-type FVB/N mice to generate F1 offspring. The F1 mice were crossed with wild-type FVB/N mice to generate F2 offspring. Mice from F1 and F2 progeny were used in this study.
Imaging pVEGF induction during wound healing. All animal experiments were reviewed and approved by the UCLA Animal Research Committee. Three- to six-week-old pVEGF-TSTA-β transgenic mice were anesthetized with ketamine-xylazine (4:1). A full-thickness wound (0.5 cm in diameter) was created by excising the dorsal skin and the underlying panniculus carnosus. The mice were serially imaged with D-luciferin (150 mg/kg body wt, ip injection) in the CCD camera on days 2 and 5 and subsequently every day until day 25. A total of six mice were studied in this experimental group. To study the correlation between the observed bioluminescence signal and endogenous VEGF induction during wound healing, the fl expression in four littermates was monitored by CCD camera (Xenogen, Alameda, CA). The four mice were killed on day 19, 20, 21, and 22, and the wound skin (1-cm diameter) and the underlying panniculus carnosus were harvested and stored frozen at −80°C. To determine endogenous VEGF gene expression in the wound region, the wound tissues were homogenized in 0.5 ml of PBS. The supernatants were used for VEGF ELISA assay using a mouse VEGF ELISA kit (R&D Systems, Minneapolis, MN). The VEGF protein level was normalized to the total protein in each sample. A total of eight mice were studied in this experimental group. To visualize the kinetics of VEGF expression at early times (days 1–4), four punch wounds each were created on days 1–4 on the back of three female transgenic mice (n = 3). The mice were imaged in the CCD camera before wounding and 4 days after wounding. The wound tissues were harvested and assayed for VEGF protein levels (ELISA).

Induction of pVEGF activity in a mouse mammary tumor model. Adult pVEGF-TSTA-β transgenic mice were anesthetized with ketamine-xylazine (4:1). NK2 cells (mouse mammary tumor; 5 × 10⁶) were injected subcutaneously on the lower right flanks of the mice. The mice were serially imaged with D-luciferin (150 mg/kg body wt, ip injection) in the CCD camera from day 3 until day 29. Four mice were imaged in this experimental group. NK2 cells (5 × 10⁶) were also injected subcutaneously on the lower right flanks of naive FVB mice. The tumor growth was closely monitored every 3 days until day 29, after which the mice were killed.

RESULTS

Construction of the pVEGF-TSTA-β system. The three single vector constructs used in this study are shown in Fig. 1A. We employed a 3-kb human pVEGF to drive the expression of a GAL4-VP2 fusion protein (containing 2 VP16 activation domains) (34). The GAL4-VP2 in turn binds to two Gal4-binding sites placed upstream of a TATA minimal promoter driving fl in a single vector (Fig. 1B). This transcriptional amplification strategy results in significant amplification of fl expression when compared with the direct one-step system (pVEGF-fl). We constructed three different plasmids and evaluated their efficacy in cell culture.

TSTA system-mediated fl expression in HeLa cells is significantly greater than the one-step system. Transient transfection of HeLa cells using the three TSTA vectors described in Fig. 1A was carried out in the absence and presence of hypoxia and cobalt chloride. The cells were also transfected with pVEGF-fl (1-step) to compare fl expression driven by the one-step system. We observe a significantly high level of FL activity under hypoxic conditions using all three plasmids. The FL induction levels for pVEGFVP2-G5-fl, pVEGFVP2G2-fl, and pVEGFVP2-G2-fl were 1.9, 3.9, and 5.6-fold, respectively, under hypoxia. We observe similar induction results using cobalt chloride. Furthermore, FL activity mediated by the TSTA system is significantly greater than that driven by the one-step system. pVEGF-fl (1,294-, 333-, and 47-fold for pVEGFVP2-G5-fl, pVEGFVP2-G2-fl, and pVEGFVP2-G2-fl, respectively) (P < 0.01) under hypoxia (Fig. 2). These results demonstrate the strong ability to titrate the levels of fl expression by varying the number of VP16 activation domains and the Gal4-binding sites. We also observed that the background FL activities in the TSTA system were also amplified under
normal conditions, and the plasmid pVEGFVP2Gz-fl showed the highest fold induction under hypoxia with minimum background. This plasmid was subsequently used to create stable cells and a transgenic mouse line.

**FL activity increases under hypoxia and cobalt chloride and demonstrates good correlation with endogenous VEGF levels.** HeLa cells stably transfected with pVEGF-VP2Gz-fl show a significant increase in FL activity when exposed to hypoxia and cobalt chloride. The FL activity postincubation increases with time up to 56 h and demonstrates an excellent correlation with endogenous VEGF levels under hypoxia (Fig. 3A, $r^2 = 0.92$) and in the presence of cobalt chloride (Fig. 3B, $r^2 = 0.96$). These results demonstrate the feasibility of indirectly measuring endogenous VEGF induction by detecting FL activity in stably transfected HeLa cells. HeLa cells stably transfected with the one-step and two-step systems were assayed for FL activity in stably transfected HeLa cells. HeLa cells stably transfected with pVEGF-TSTA-fl expression is highly upregulated during wound healing and demonstrates a good correlation with the one-step system (data not shown).

**pVEGF-driven fl expression is highly upregulated during wound healing and demonstrates a good correlation with endogenous VEGF protein levels.** The pVEGF-TSTA-fl transgenic mice were imaged before creation of a wound to obtain basal bioluminescence signal. Color images of visible light are superimposed on photographic images of mice with a scale in photons per second per square centimeter per steradian (p.s$^{-1}$cm$^{-2}$sr$^{-1}$). After wound creation, the bioluminescence signal emitted from the wound lesion was detected on days 1–4 after wounding using a mouse VEGF ELISA assay kit. VEGF protein expression in the wound tissue was measured in the early days (days 1–4) after wounding using a mouse VEGF ELISA assay. VEGF protein expression in the wound tissue was found to increase from day 1 to day 29 (34.0 ± 5.0, 54.0 ± 6.0, 51.0 ± 6.0, and 118.0 ± 21.0 pg VEGF/µg protein, averaged across 3 mice, $P < 0.05$; Fig. 5). The control sample (obtained using the bioluminescence signal in a sample of normal skin) had a VEGF protein level of 18.4 ± 8.4 pg VEGF/µg protein. Although there was evidence of increasing VEGF expression on days 1–4, bioluminescence signal was detected in the wound area only on day 4. The lack of bioluminescence signal during the early days is likely due to several factors. These include substrate delivery (not enough substrate reaching the target site), relatively low light output due to low levels of transcriptional activation, and low light transmitted due to absorption and scattering (22). Nevertheless, these findings suggest that in vivo bioluminescence imaging can be used to monitor VEGF expression in vivo a few days after skin wounding.

**VEGF induction in transgenic mice can be monitored using a subcutaneous mammary tumor model.** The pVEGF-TSTA-fl mice were implanted with NK2 cells in the right bottom flank and subsequently imaged in the CCD camera from day 3 until day 29. Figure 6A shows the longitudinal monitoring of pVEGF-induced fl expression in the area around the tumor on days 3, 8, 10, 15, 17, 22, 24, and 29. Bioluminescence signal is detected on day 3 (1.1 × 10$^6$ p.s$^{-1}$cm$^{-2}$sr$^{-1}$) and continues to increase until day 17 (5.8 × 10$^6$ p.s$^{-1}$cm$^{-2}$sr$^{-1}$) followed by a considerable decline on days 22 (1.4 × 10$^6$ p.s$^{-1}$cm$^{-2}$sr$^{-1}$) and 29 (2.8 × 10$^6$ p.s$^{-1}$cm$^{-2}$sr$^{-1}$). The tumors started to regress after 2 wk, and the bioluminescence signal also showed a corresponding decline. The initial increase in signal from day 4 to day 17 is 5-fold, and the subsequent decrease on days 22 and 29 is 4-fold and 20-fold, respectively. Figure 6B shows a bar graph of the maximum photon counts in the area around the tumor (averaged across 4 mice) on different days during the imaging period. To study

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**Fig. 3.** Correlation between VEGF levels and FL activities in HeLa cells stably transfected with pVEGFVP2Gz-fl under hypoxia (A) and cobalt chloride conditions (B). Cells were assayed for FL activity at different time points (6, 24, 32, 48, and 56 h, as described in MATERIALS AND METHODS).
whether the tumor regression was specific to pVEGF-TSTA-fl transgenic mice, we injected wild-type FVB mice with NK2 cells and followed the tumor growth until day 29. We observed that the kinetics of tumor growth were similar to that observed in the transgenic mice. The tumor continued to grow until day 14 and then started to regress. By day 29, there was no visible tumor. Tumor regression with NK2 cells in FVB mice has also been observed by others (personal communication with Dr. Tim Lane at UCLA).

pVEGF-TSTA-fl transgenic mice display normal physical characteristics with no observed deleterious effects from the transactivator. The GAL4-VP16 transactivation system is a powerful tool for amplifying transgene expression from a weak promoter. However, it has been reported that high levels of the transactivator can be detrimental to cells (29). In the present study, comparison of litter weights, growth rates, and weaning weights indicated no abnormal differences between the transgenic and wild-type mice. Recently, we have reported the development and imaging of a prostate-specific TSTA transgenic mouse model (15). We did not observe any deleterious effects of the transactivator on the progeny of the TSTA or pVEGF-TSTA-fl transgenic mice.

DISCUSSION

Noninvasive imaging of VEGF activation during wound healing and tumor growth will provide a better understanding of the role of VEGF under these conditions. In the current

Fig. 4. Induction of fl expression during wound healing in a pVEGF-TSTA-fl transgenic mouse. A: mouse was imaged before wound creation in the CCD camera (day 0) and imaged again subsequent to wound creation every 4–5 days using D-luciferin (150 mg/kg ip). B: optical CCD images of four pVEGF-TSTA-fl transgenic mice (mice 1–4) on days 19, 20, 21, and 22 after creation of the wound. Arrows indicate the position of the wounds. C: correlation plot of maximum bioluminescence signal (p/s/cm²/sr) vs. endogenous VEGF levels in the wound tissue (r² = 0.70). Error bars represent the standard error for triplicate samples in the ELISA assay.
work, the full-length human pVEGF was used to drive fl reporter gene expression using a TSTA strategy. Endogenous VEGF activation was accomplished in cell culture using cobalt chloride and hypoxia and in vivo using a wound-healing model and a mouse mammary tumor model in pVEGF-TSTA-fl transgenic mice. VEGF activation as visualized by fl expression was clearly demonstrated in both the wound lesion and growing tumor for up to several days. The bioluminescence signal in the tumor continued to increase until day 17, followed by a decrease that was maintained up to the end of the imaging period. A similar trend was observed in all mice studied. It is likely that the bioluminescence signal around the tumor site is the result of VEGF induction in the surrounding stroma. We studied VEGF expression in the NK2 cells and found that the cells express VEGF at high levels. However, because these cells do not carry the fl reporter gene, the bioluminescence signal observed in the area around the tumor in the days after cell implantation can be attributed to VEGF induction in the stroma. The VEGF that is made by the tumor cells does not lead to bioluminescence in the current model. The VEGF induction can also in part be from inflammatory cells. Future studies can include models in which the tumor expression of VEGF is monitored by a separate reporter gene while the current model measures stromal activation of VEGF. The spatial resolution in the current imaging does not allow us to distinguish stromal cells that may have infiltrated the tumor from the tumor itself. Evidence from a previous study suggests that the most prominent cells showing VEGF promoter activation are spindle-shaped cells that display structural features of fibroblasts (10). Future studies with this transgenic model will need to look at the cell types involved in signal production. We observed a decrease in tumor size after 14 days with a corresponding decrease in the bioluminescence signal. The regression in tumor size was observed not only with the pVEGF-TSTA-fl transgenic mice but also in naïve FVB mice injected with NK2 cells. In the wound-healing experiments, pVEGF-induced fl expression in the wound area was detected on day 5, and peak bioluminescence signal was observed on days ranging from 15 to 22. The bioluminescence signal showed a good correlation with the endogenous VEGF protein levels in the wound tissue on days 5–21 (r² = 0.70). VEGF protein levels

Fig. 5. Kinetics of VEGF expression at early times after skin wounding. Four punch wounds each were created on days 1–4 on the back of three female transgenic mice (n = 3). The mice were imaged in the CCD camera before wounding and 4 days after wounding. On the 5th day, the wound tissues were harvested and assayed for VEGF protein levels (ELISA).

Fig. 6. Longitudinal monitoring of VEGF promoter activity in pVEGF-TSTA-fl transgenic mice using a mouse mammary tumor model. A: pVEGF-TSTA-fl transgenic mice were injected subcutaneously with 5 × 10⁶ NK2 cells in the lower right flank. Mice were imaged on day 4 and subsequently every few days until day 29 using β-luciferin as the reporter substrate (150 mg/kg, injected ip). CCD camera imaging revealed a detectable bioluminescence signal in the area around the tumor on day 4. Signal intensity continued to increase until day 17, after which a considerable decline in signal was observed (days 22–29). B: bar graph of maximum photons in the area around the tumor (p·s⁻¹·cm⁻²·sr⁻¹) during different days of imaging. Data were averaged across four mice.
measured in the early days after skin wounding continued to increase from day 1 to day 4. However, bioluminescence signal was detected in the wound tissue only on day 4. The absence of a signal in the early days may be related to the amount of substrate (o-luciferin) reaching the target site and absorption and scattering of light leading to a relatively low light output. In the current study, we used a tumor cell line that regressed with time. Future studies using nonregressing cell lines will be helpful in further characterizing this transgenic model. This study demonstrates that the imaging strategies employed allow noninvasive and repetitive monitoring of endogenous VEGF gene expression during wound healing and tumor progression through the use of a noninvasive reporter gene assay with excellent correlations in cell culture and good correlations in living transgenic mice.

Our initial attempts to drive the reporter gene directly with the pVEGF were not robust enough for imaging in living mice. Although gene induction using the one-step system was possible, the absolute level of reporter activity was found to be very low. For this reason, we employed the TSTA system that we have previously validated (17, 35) to help overcome the relatively weak transcriptional activity of pVEGF. This led to significant gene induction and a high absolute reporter signal after induction, but at the expense of a higher background signal before induction. The absolute levels of reporter gene expression varied based on the choice of the V2G2, V1G5, or V2G5 TSTA strategies (Fig. 2). We chose the V2G2 TSTA system for the current transgenic model because we wanted to maximize the induced fl bioluminescent signal while not increasing the background preinduction signal too much. The background signal, due to basal levels of reporter gene expression before induction of pVEGF, did not pose a significant problem because it is still relatively low for in vivo imaging studies compared with the signal after gene induction. The background signal for V1G5 and V2G5 is considerably higher based on the results in Fig. 2. Future studies may be able to further optimize maximizing the induced reporter signal while minimizing the preinduction background signal.

A key goal in developing the transgenic model for this work was to allow for noninvasive monitoring of endogenous VEGF gene expression. A single previous study has used gfp as a reporter (10) to monitor VEGF levels but failed to demonstrate whether the qualitative signal correlates with endogenous VEGF gene expression. In fact, no attempts to relate the signal to endogenous VEGF gene expression were undertaken in that work. Furthermore, gfp has some specific limitations for applications in living subjects because of poor depth penetration and background autofluorescence (2). The development of newer and more advanced techniques such as fluorescence-mediated optical tomography and multichannel imaging may lead to considerable improvements in relation to depth penetration and quantitation. Improvements in BLI, including BLI tomography, may also help quantitation and depth information when using the transgenic developed in this study. The current mouse model incorporating amplified promoter activity and bioluminescence imaging should be very useful to the research community. Recently, Zhang et al. (36) have reported the kinetics of VEGF receptor type 2 (VEGFR2) gene expression in different pathophysiological processes using BLI (36). The authors reported the generation of a transgenic mouse model wherein fl reporter gene expression is driven by a murine VEGFR2 promoter. The authors observed induction of VEGFR2/fl expression in endothelial cells during wound healing. The VEGFR2 transgenic model represents a one-step system where a 0.5-kb fragment from the first VEGFR2 intron was placed downstream of the fl gene. This led to increased VEGFR2 expression in endothelial cells. The transgenic developed in this work allows the study of VEGF gene expression, whereas the above study allows the study of VEGFR2 expression, and both models should complement each other. In the present study, the use of the fl reporter coupled with the TSTA strategy allows very low background signal and highly sensitive detection at all depths throughout the mouse. Although in the current work we have focused on superficial wounds, the strategies developed should also facilitate signal detection at any depth within rodents. In the current work, we successfully demonstrated that the reporter signal correlates both in cell culture ($r^2 > 0.90$) and in living transgenic mice ($r^2 = 0.70$) with endogenous VEGF gene expression. The correlation was not as high in vivo as it was in cell culture, likely reflecting that it is harder to measure absolute regional VEGF levels in the wound and limitations due to heterogeneity within a given region. Future studies will have to further explore this correlation with more animals under more experimental conditions in addition to wound healing. Also, additional studies to look at serum VEGF in addition to regional tissue VEGF should add further utility.

The TSTA strategy has been used by us previously to create a transgenic model for prostate-specific expression (16). In that model and in the current model, we did not observe any gross toxicity with expression of GAL4 and VP16. Although there may be some subtle effects related to expression of these foreign genes, we did not observe anything at a more gross level in the current study. Future studies will need to continue to explore any potential toxicity of this system in transgenic mice models. An ideal system would enable monitoring of VEGF activation in any tissue at any depth with high sensitivity in a living intact subject. In this study, we have developed a transgenic mouse that allows noninvasive assessment of VEGF in various tissues and has a slightly poorer level of detection compared with direct tissue biopsy. The key advantage is the ability to serially monitor the same mouse over time to study changes in VEGF activation. This transgenic mouse model can potentially be useful in evaluating in vivo any new drugs that directly or indirectly modulate VEGF transcriptional activation. Molecular imaging of living subjects is allowing many applications in which therapies can be tested in vivo. This allows optimization of in vivo pharmacokinetics and can accelerate drug testing in preclinical models. In addition, this mouse can be mated with mice carrying other reporters to study activation of multiple genes. For example, we are now studying the interaction of expression of the Cox-2 gene with the VEGF gene by mating the two appropriate mice carrying different reporters. As many different reporter mice are developed with different pathways that can be monitored, this will likely lead to insights into cell biology and testing of novel therapies. We therefore feel this mouse model will be useful for many different purposes.

Although currently studied with a bioluminescent reporter, future transgenics should be able to be developed with multimodality tri-fusion reporters (27, 28). This would allow imaging of the same animal using fluorescence, bioluminescence,
and PET. This will likely provide greater tomographic and quantitative detail than the current model. With continued refinement and exploration of other models, the VEGF transgenic mouse model described in the present study should be useful to investigators for many different applications in which repetitive monitoring of endogenous VEGF gene expression is desired.

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