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14. ABSTRACT Breast cancer growth relies on a blood supply. Therefore, anticancer therapies that disrupt tumor angiogenesis to starve tumor cells can be very effective. To examine the importance of candidate proteins in adult angiogenesis, we generated a transgenic mouse model expressing the receptor for an avian retrovirus only in new blood vessels. The avian retrovirus was en-gineered to express ephrin-B2 (a protein whose importance in blood vessel growth we wanted to examine) fused to green fluorescent protein (EGFP) and, as controls, EGFP alone and an onco-gene known to promote endothelial cell proliferation also fused to EGFP. In vivo angiogenesis assays and tumor analysis demonstrated expression of the retroviral receptor in new blood vessels and susceptibility to infection by the retrovirus causing expression of the EGFP-tagged proteins. However, optimization of virus delivery to obtain higher and more consistent levels of infection will be needed to establish if a protein has pro-angiogenic activity. The mouse model generated, which will also be useful to study the role of proteins in					
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Introduction

Ephrin-B2 is a transmembrane protein expressed in endothelial cells. Recent evidence indicates that ephrin-B2 is required for the formation of functional blood vessels in the embryo (Wang et al., 1998). Ephrin-B2 is also expressed at sites of adult neovascularization, including tumor blood vessels (Gale et al., 2001; Shin et al., 2001; Noren et al., 2004). Interaction of ephrin-B2 with EphB4 and other EphB receptors at sites of cell-cell contact generates signals both in cells expressing the EphB receptor and in cells expressing ephrin-B2. The hypothesis underlying this project was that ephrin-B2 promotes the formation of a functional blood circulation in tumors. In support of this hypothesis, we and others recently demonstrated that promoting ephrin-B2 signaling in tumor blood vessels through upregulation of the EphB4 receptor in tumor cells or in endothelial cells dramatically increases tumor growth (Noren et al., 2004; Erber et al., 2006). Upregulation of ephrin-B2 expression in tumor endothelial cells should also promote tumor angiogenesis and, as a consequence, breast cancer growth while inhibiting ephrin-B2 expression or signaling should inhibit tumor angiogenesis. Interestingly, ephrin-B2 expression in endothelial cells is upregulated by hypoxia and vascular endothelial growth factor (VEGF), two factors that have been implicated in tumor angiogenesis (Mukouyama et al., 2002; Suenobu et al., 2002).

To test the involvement of ephrin-B2 (and other proteins of interest) in pathological forms of adult angiogenesis, we proposed to generate transgenic mice that express the TVA receptor for the RCAS avian retrovirus under the control of Flk1 promoter and enhancer sequences. Flk1 is a receptor for VEGF that is expressed in the embryonic and angiogenic vasculature, but not in quiescent adult vasculature (Kappel et al., 1999). The Flk1 promoter/enhancer was therefore expected to selectively drive TVA receptor expression in endothelial cells of newly forming blood vessels in the transgenic mice. As demonstrated in previously generated TVA transgenic models, mouse cells that express the TVA receptor on their surface acquire susceptibility to infection by the RCAS virus, which can be engineered to encode a protein of interest (Fisher et al., 1999; Orsulic, 2002). We engineered the RCAS retrovirus to express ephrin-B2, and the Polyoma Middle T (PyMT) oncogene as a control, in the endothelial cells of forming blood vessels. These proteins were tagged with green fluorescent protein (EGFP), which allowed us to monitor their expression. The TVA transgenic mice were used for *in vivo* Matrigel "plug" angiogenesis assays and crossed with transgenic mice that develop multifocal, metastatic mammary adenocarcinoma with a short latency. These experiments validated the newly developed TVA transgenic mouse model but were only suggestive of pro-angiogenic effects on endothelial cells, given the low and variable levels of endothelial cell infection achieved. After further optimization of infection conditions, the Flk1-TVA mouse model will be useful to evaluate whether proteins expressed in adult neovasculature play a functional role in angiogenesis and are, therefore, promising targets for anti-cancer and anti-angiogenic therapies. The mice are also suitable to study proteins involved in the differentiation/proliferation of Flk1-positive hematopoietic progenitor cells, which will also be infectable with engineered RCAS viruses. The TVA transgenic mice generated in the course of this project will be made available to the scientific community.

Body

Task 1. Generate transgenic mice that selectively express the TVA receptor for the RCAS retrovirus in new vasculature

1.a. Generate TVA transgenic mice. We obtained the Flk1 promoter/enhancer sequences in the pGL2-Flk1-lacZ plasmid from Dr. Georg Breier (Max Plank Institute, Germany) (Kappel et al., 1999). We obtained the TVA cDNA from Dr. Varmus (NIH laboratories) (Bates et al., 1993) and subcloned it into the pGL2-Flk1 vector in place of LacZ to obtain the pGL2-Flk1-TVA plasmid. The Flk1-TVA portion was isolated from the vector backbone by digestion with restriction enzymes, gel purified, and used to microinject fertilized oocytes obtained from FVBN female mice. Following microinjection, the oocytes were transferred to the oviducts of pseudopregnant Swiss Webster mice. Identification of suitable carriers was done using genomic DNA extracted from tail clippings and PCR with primers designed to amplify the TVA gene (see Fig. 7 below). The microinjection experiments worked extremely well and we identified 15 transgenic lines.

1.b. Construct the RCAS-ephrin-B2-EGFP vector. We used the RCAS-EGFP control virus that was already available in the laboratory and obtained RCAS- β -galactosidase virus from Dr. Yi Li (Memorial Sloan-Kettering Cancer Center) as an additional control. We also engineered an RCAS construct encoding full length ephrin-B2 fused to EGFP (RCAS-ephrin-B2-EGFP) (Fig. 1). We reasoned that since the infection levels of endothelial cells in Matrigel plugs appeared to be low and inconsistent, it would be easier to detect enhanced vascularization due to increased ephrin-B2 signaling rather than decreased vascularization due to inhibition of ephrin-B2 signaling by the dominant negative approach originally proposed. Cells expressing increased levels of ephrin-B2 would also be expected to more readily proliferate (Noren et al., 2004), further facilitating their detection. Increasing or decreasing ephrin-B2 function are both viable approaches to evaluate the importance of ephrin-B2 in adult neovascularization. Preparation of RCAS encoding full length ephrin-B2-EGFP required a more complicated cloning strategy than initially anticipated because an internal ClaI site in the ephrin-B2 cDNA sequence had to be mutated to allow insertion of the ephrin-B2-EGFP cDNA into the only cloning site of RCAS, which is a ClaI cloning site. Although not initially planned, we also successfully engineered an RCAS virus containing a fusion between EGFP and the Polyoma Middle T (PyMT) oncogene as an additional control. The advantage of this RCAS construct is that it encodes an oncogene known to strongly stimulate endothelial cell proliferation (Bautch et al., 1987), which should enlarge the population of infected endothelial cells and make infection easier to detect (see Task 2 below).

1.c. Produce infective RCAS viruses. The different RCAS viruses were produced in the DF1 chicken cell line. Briefly, DF1 cells were transfected with the RCAS plasmids, allowed to reach confluency, and subcultured to obtain nearly 100% infection. Infection levels were monitored through the green fluorescence (Fig. 1) or β -galactosidase marker. Virus was concentrated from culture medium by ultracentrifugation and titered by infecting DF1 cells and counting cells that express EGFP or β -galactosidase 48 hr later. We obtained titers of 10^6 - 10^8 infectious units/ml for the different viruses.

Task 2. Use the RCAS retrovirus to express dominant negative ephrin-B2 in the neovasculature of TVA transgenic mice to determine if ephrin-B2 signaling is required for angiogenesis

As outlined above and in previous annual reports, difficulties in detecting *in vivo* endothelial cell infection prompted us to slightly modify our initial approach aimed at demonstrating the involvement of ephrin-B2 in adult angiogenesis. We decided to evaluate whether full length ephrin-B2 promotes angiogenesis instead of evaluating whether dominant negative ephrin-B2 inhibits it. Additional, but equally important, goals of these experiments were to identify the best Flk1-TVA transgenic lines and validate the Flk1-TVA transgenic model, which could be useful to study the angiogenic role of many different proteins.

2.a. Verify that the RCAS virus infects the neovasculature of TVA mice. This subtask consisted of first identifying the transgenic lines that expressed the highest levels of TVA mRNA and protein in newly formed blood vessels and then examining the susceptibility of the new blood vessels to RCAS infection.

2.a.1. Verify expression of TVA mRNA and protein. We decided to screen the mouse lines for TVA receptor expression before testing for their susceptibility to infection by the RCAS virus because we obtained so many lines, and many transgene integration sites may not permit robust expression. Furthermore, TVA expression should be detected in all new blood vessels whereas only a few endothelial cells may be infected by RCAS. To assess overall expression of the TVA mRNA, we developed an RT-PCR assay with postnatal day 3 to 5 (P3-P5) brains because blood vessels in the brain have been reported to maintain substantial Flk1 expression until early postnatal ages (Fig. 2) (Millauer et al., 1993). We expected the TVA receptor to have a similar expression pattern as Flk1 since it is under the control of the same regulatory sequences. We found that 12 of the 15 lines expressed detectable TVA transcripts (Table 1). Therefore, we maintained and continued analyzing those 12 lines.

High overall TVA mRNA expression could be indicative of either high expression in endothelial cells or low widespread expression in different types of cells because some transgene integration sites may cause widespread expression. Therefore, we screened embryos at days 10 to 11 of development (E10-E11) from different transgenic lines by whole mount *in situ* hybridization for the TVA receptor (Fig. 3). *In situ* hybridization for the Flk1 receptor was used as a comparison because TVA expression in the transgenic mice is under the control of Flk1 regulatory elements (Kappel et al., 1999). The *in situ* hybridization results allowed us to identify six transgenic lines with high and selective TVA expression in the developing vasculature (Table 1). In most lines there was a good correlation between the levels of overall TVA expression in P3-P5 brain and expression in the embryonic vasculature.

It was also important to assess the levels of TVA protein expression, since it is the interaction of the TVA receptor protein with the RCAS virus that mediates viral entry into endothelial cells leading to infection. We prepared affinity-purified anti-TVA antibodies by immunizing rabbits with a GST-TVA fusion protein (Bates et al., 1993). These antibodies showed strong specific labeling in control experiments in which we stained paraffin sections of 293 human embryonal kidney cells embedded in Matrigel (an extract of murine basement membrane proteins, see below) (Fig. 4). We then used the anti-TVA antibodies to identify the transgenic lines with highest TVA protein expression in new adult vasculature, as described in the next section.

2.a.2. Establish the "Matrigel plug" assay. To examine TVA protein expression in new adult vasculature, we used the "Matrigel plug" assay (Passaniti et al., 1992). Briefly, Matrigel was injected subcutaneously in TVA transgenic mice and control non-transgenic mice. The Matrigel was kept at 4°C prior to injection because it is liquid at this temperature. At body temperature the injected mixture forms a gel that persists for at least 10 days. To promote the growth of new blood vessels into the plugs, the Matrigel was supplemented with bFGF and heparin or with VEGF. When we used RCAS-ephrin-B2-EGFP, we also supplemented the Matrigel with the EphB4 extracellular domain fused to the Fc fragment of human IgG₁ (EphB4 Fc). EphB4, which is a receptor for ephrin-B2 and stimulates ephrin-B2 signaling, was used to enhance migration of the infected endothelial cells expressing high levels of ephrin-B2 into the Matrigel (Noren et al., 2004). We stained with the anti-TVA antibodies Matrigel plugs that were implanted in the 6 transgenic lines found positive by *in situ* hybridization. Endothelial cells showed strong TVA immunoreactivity in line 1026 (Fig. 5). The second best line was 1029, in which TVA immunoreactivity in endothelial cells was clearly detectable but lower and more sporadic than in the 1026 line (Fig. 5). Low TVA expression was also detected in line 14 (not shown). TVA immunoreactivity in the remaining 3 transgenic lines was very low to undetectable. Immunoreactivity in the 1026 line was present in all endothelial cells (identified by double-labeling with CD31 antibodies) and absent in other cell types (CD31-negative) present in the plugs (Fig. 5).

2.a.3. Evaluate the susceptibility of new blood vessels to RCAS infection. We also used the Matrigel plug assay to determine whether the new vasculature of mice from the different lines could be infected by the RCAS virus. We used the RCAS-EGFP control virus that was already available in the laboratory for initial experiments. RCAS-EGFP control virus was mixed with the Matrigel at the time of injection. Matrigel plugs were removed 7 to 10 days later and frozen. Initially, we analyzed frozen sections for EGFP fluorescence. We could detect only few green fluorescent cells in only some of the experiments, and non-infected cells in the plugs had substantial background endogenous fluorescence that made it difficult to unequivocally identify the infected cells. Therefore, we performed immunohistochemistry with anti-EGFP antibodies to enhance the signal. Another advantage of the immunocytochemistry approach compared to direct detection of green fluorescence was the ability to double-label the sections with anti-CD31 antibodies in order to identify the endothelial cells among the different cell types in the Matrigel plugs.

We detected EGFP-expressing cells in the Matrigel plugs containing RCAS-EGFP virus but not in the plugs without virus (not shown). However, the results were variable and when infected endothelial cells could be visualized, their number was low. We also tried RCAS- β -galactosidase virus because β -galactosidase enzymatic activity is more easily detectable than EGFP fluorescence. However, the cDNA encoding β -galactosidase is quite large, and therefore a drawback was that the corresponding RCAS virus cannot be produced at high titers. Therefore, we decided to use RCAS encoding EGFP-PyMT, an oncogene known to promote endothelial cell proliferation (Bautch et al., 1987), in order to more easily detect infection. Use of RCAS-EGFP-PyMT virus resulted in a larger fraction of EGFP-expressing endothelial cells, which were either infected by the virus or derived by proliferation of infected cells (Fig. 6). Infection was more consistently detected in mice from the 1026 and 1029 lines, which were the ones with the highest levels of TVA protein expression. RCAS infection was also sporadically detected in line 14. However, even in line 1026 only a small fraction of the endothelial cells was infected.

Task 3. Cross the TVA mice with transgenic mice that develop mammary tumors to determine if ephrin-B2 signaling is required for tumor vascularization and growth

3.a. Generate TVA-middle T bigenic mice. TVA mice of line 1026, which had highest TVA protein expression and highest infection of endothelial cells in Matrigel plugs, were crossed with MMTV-PyMT transgenic mice in collaboration with Dr. Robert Oshima (Fig. 7). These mice express the Polyoma Middle T oncogene in mammary epithelial cells under the control of the mammary tumor virus-long terminal repeat (MMTV-LTR) sequences. We used these mice, which develop mammary tumors, in order to screen for TVA receptor expression and susceptibility of the tumor blood vessels to infection with RCAS virus. In addition, we developed in collaboration with Dr. Oshima a method to inject RCAS virus in a section of vasculature near a mammary tumor. For each tumor, the virus was injected into a large vein extending collateral branches into the tumor. The extremities of the injected vein were closed off, allowing the virus to remain in the vessel branches within the tumor for a prolonged period rather than being rapidly removed by the circulating blood.

By immunohistochemistry with anti-TVA antibodies, we detected TVA receptor in only a subset of blood vessels, preferentially at the periphery of the tumor (Fig. 8). Presumably those represent the newly forming blood vessels. Infection experiments were carried out using RCAS-EGFP-PyMT virus, to maximize our ability to detect infected endothelial cells and optimize viral delivery. In some experiments, we also injected RCAS-EGFP. EGFP immunoreactivity was detected in only a small subset of endothelial cells and only in the tumors infected with RCAS-EGFP-PyMT (Fig. 9). This result is encouraging and consistent with an expansion of the infected endothelial cells that express the PyMT oncogene. However, given the low levels of infection, other approaches to deliver the RCAS virus will have to be tried to allow reaching reliable conclusions on the effects of a protein on mammary tumor angiogenesis. These approaches may include intravenous or intraperitoneal virus delivery (Montaner et al., 2003; Montaner et al., 2004), which will however require large quantities of virus in adult mice. Detecting the effects of an angiogenesis inhibitor will probably be difficult with this approach, unless much higher infection levels can be achieved. However, our results with the RCAS-PyMT virus predict that this mouse model is suitable for assessing pro-angiogenic effects.

Table 1. TVA Transgenic Lines

Transgenic line	RT-PCR P3-P5 brain	<i>in situ</i> E10-E11 embryos	Endothelial infection in Matrigel plugs	Endothelial infection in Middle T tumor model
1026	++++	positive	positive	positive
1017	++++	positive	negative	
1008*	++++	positive	negative	
1041	++++	positive	negative	
1029	+++	positive	positive	
14	+++	positive	positive	
1014	++	negative		
1021	++	negative		
1004	+	negative		
1005	+	negative		
1053	+	negative		
1**	+++			
1060**	++			
12***	++			
1002	-			

*The transgene was unstable; **problems breeding; ***two distinct integration sites.

The two lines that performed best in all the assays are highlighted in gray.

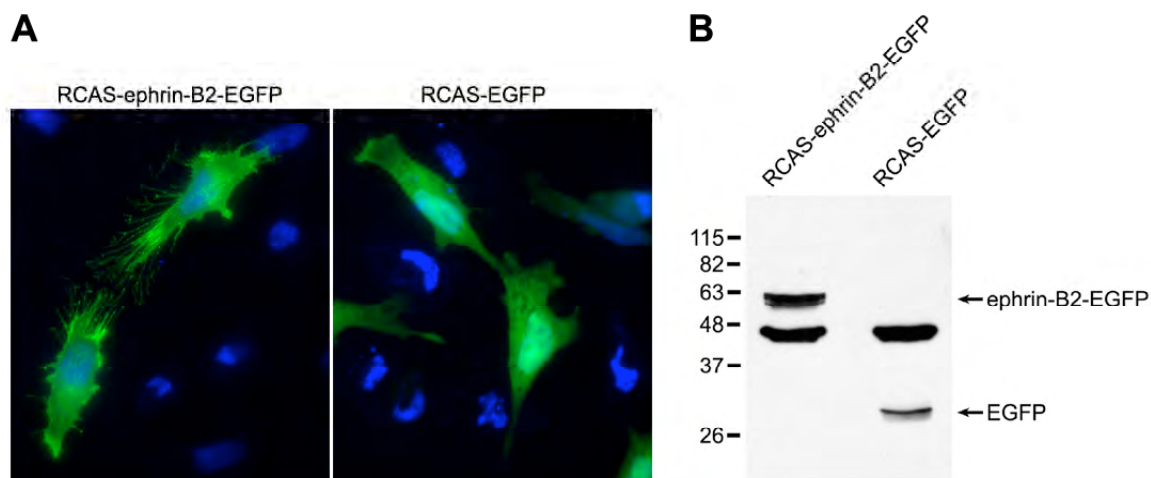


Fig. 1. RCAS-ephrin-B2-EGFP. The DF1 chicken cell line was transfected with either RCAS-ephrin-B2-EGFP or RCAS-EGFP viruses. (A) Transfected cells express the protein encoded by the RCAS virus, as shown by their green fluorescence. The nuclei of all cells were stained with DAPI (blue). Note the altered cell morphology induced by ephrin-B2 expression. (B) Immunoblotting with anti-EGFP antibodies shows proteins of the appropriate molecular weights for ephrin-B2-EGFP and EGFP. An irrelevant background band at 45 Kd is also detectable in both lanes.

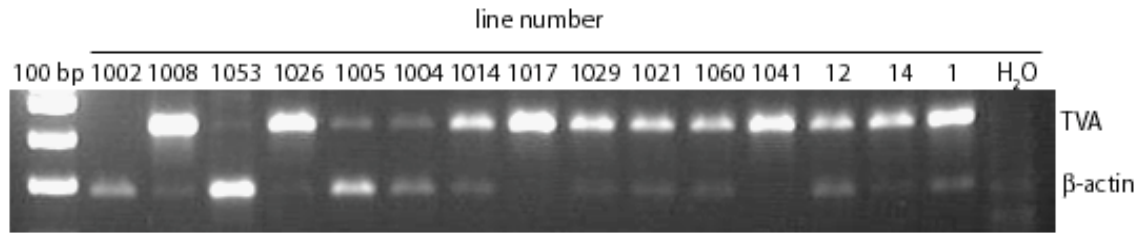


Fig. 2. TVA mRNA expression in the transgenic mouse lines. mRNAs prepared from P3 brains of transgenic mice from different lines were used for reverse transcription and PCR amplification with TVA specific primers and β -actin primers. As a negative control, the PCR reaction was carried out without cDNA (H_2O). Comparison of the TVA signal and the β -actin signal gives an estimate of the overall levels of TVA mRNA expression in the tissue.

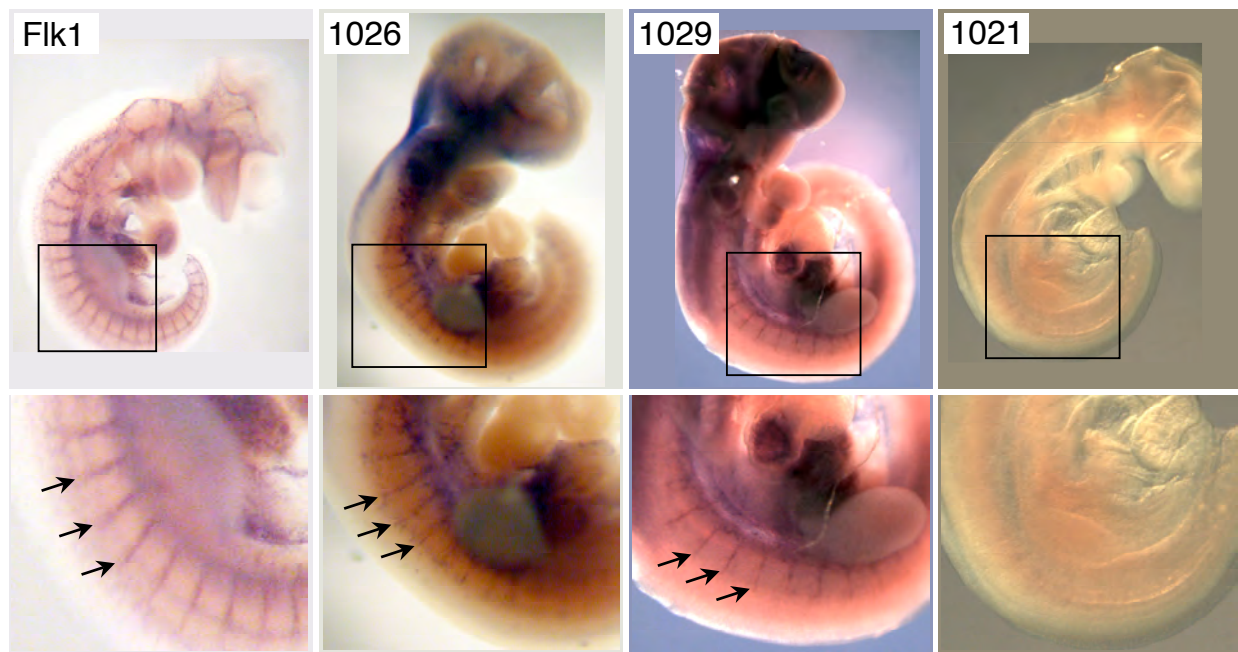


Fig. 3. Screening of the transgenic mouse lines by *in situ* hybridization for TVA receptor expression. Whole-mount E10.5 mouse embryos from the 1026, 1029 and 1021 transgenic lines were hybridized with a 389 bp TVA antisense probe. Because TVA expression is driven by Flk1 regulatory elements, the staining pattern was compared to that of Flk1 as a positive control. Lines 1026 and 1029, but not 1021, express TVA in structures where Flk1 is also expressed, including intersomitic blood vessels (arrows).

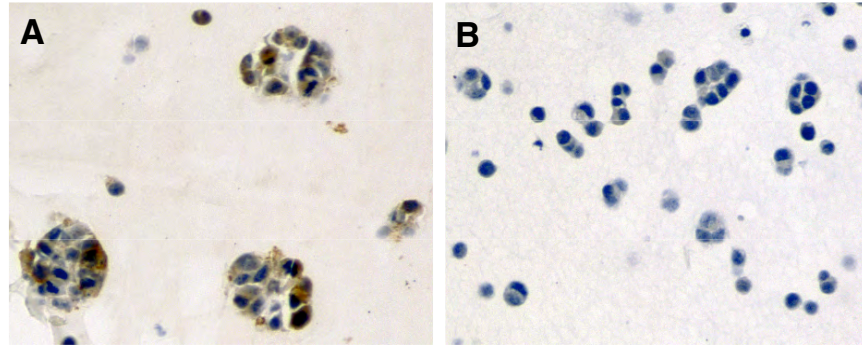


Fig. 4. Affinity purified anti-TVA antibodies label cells expressing the TVA receptor. Anti-TVA antibodies were used to stain 293 cells transfected with the TVA receptor (A) or untransfected 293 cells (B). Cells were embedded in liquid Matrigel, and the solidified Matrigel was fixed in 4% paraformaldehyde, paraffin embedded and processed for horseradish peroxidase immunocytochemistry. Peroxidase staining for TVA is brown. Sections were counterstained with hematoxylin (blue) to visualize nuclei.

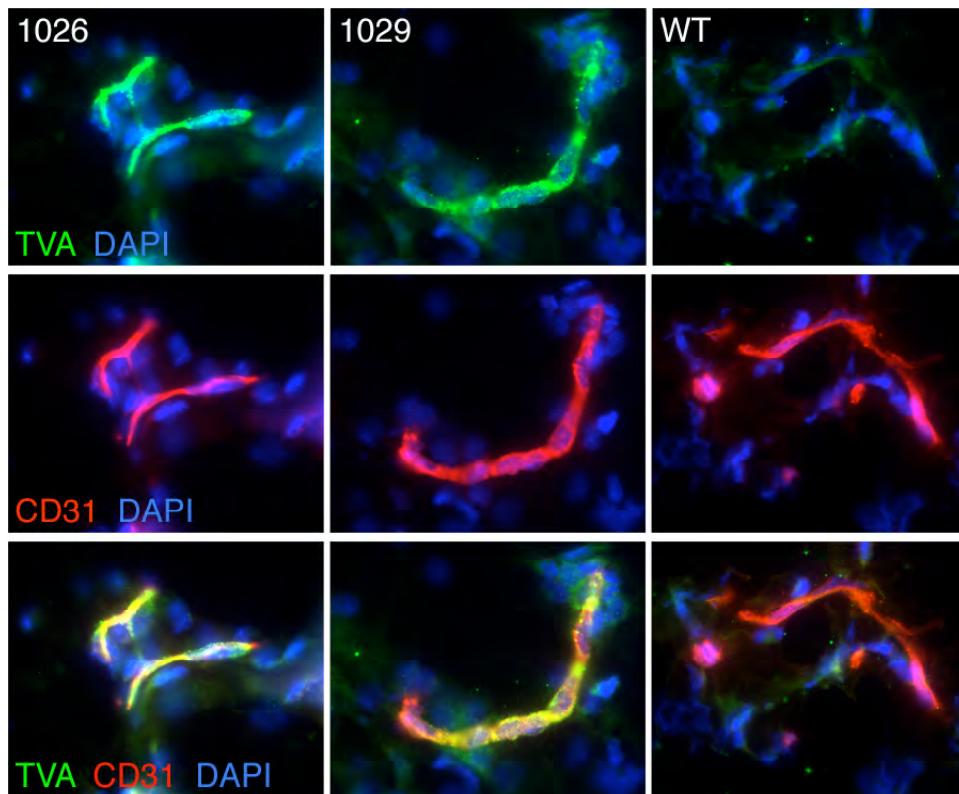


Fig. 5. *In vivo* Matrigel assay to determine expression of the TVA receptor in new endothelial cells of Flk1-TVA transgenic mouse lines. Matrigel supplemented with VEGF, EphB4 Fc and RCAS-ephrin-B2-EGFP virus was injected subcutaneously in the mice. The Matrigel plugs were removed at day 7, frozen and sections were stained with anti-TVA antibodies to detect cells expressing the TVA receptor (green), the endothelial-specific marker CD31 to visualize blood vessels (red), and DAPI to visualize the nuclei of all cells present (blue). All pictures were taken at the same exposure and identically adjusted in Photoshop. Note that all endothelial cells are stained with the TVA antibodies in the 1026 and 1029 transgenic lines but not in wild type (WT) mice.

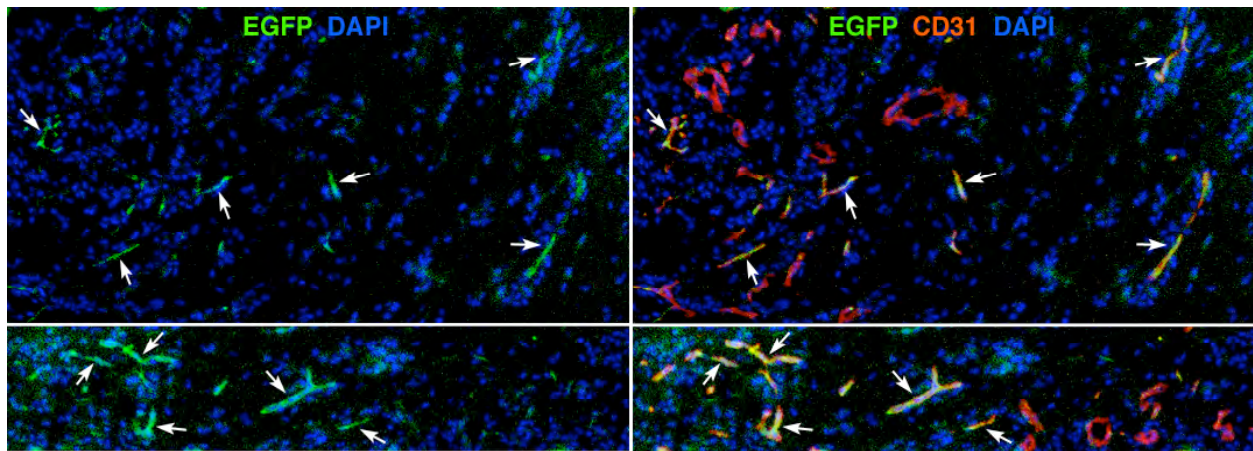


Fig. 6. *In vivo* Matrigel assay to determine susceptibility of the TVA transgenic lines to infection by the RCAS retrovirus. Matrigel supplemented with bFGF and heparin was injected subcutaneously in mice of line 1026. To determine whether RCAS can infect the new vasculature that grows into the Matrigel plugs, RCAS virus encoding the polyoma middle T antigen (PyMT) was mixed with the Matrigel at the time of injection. PyMT was used to enhance proliferation of the infected cells, and was tagged with enhanced green fluorescent protein (EGFP) as a marker to identify the infected cells. The Matrigel plugs were removed at day 10, frozen and sections were double-labeled with anti-EGFP antibodies (green) and anti-CD31 antibodies to label endothelial cells (red). DAPI (blue) was used to stain cell nuclei. The pictures, showing two example of the staining, were taken at the same exposure and identically adjusted in Photoshop. Arrows point to blood vessels labeled by the anti-EGFP antibodies and therefore infected with the RCAS virus. Note that only a subset of the endothelial cells is infected while the others are labeled only by the anti-CD31 antibodies (red), suggesting that they are not infected.

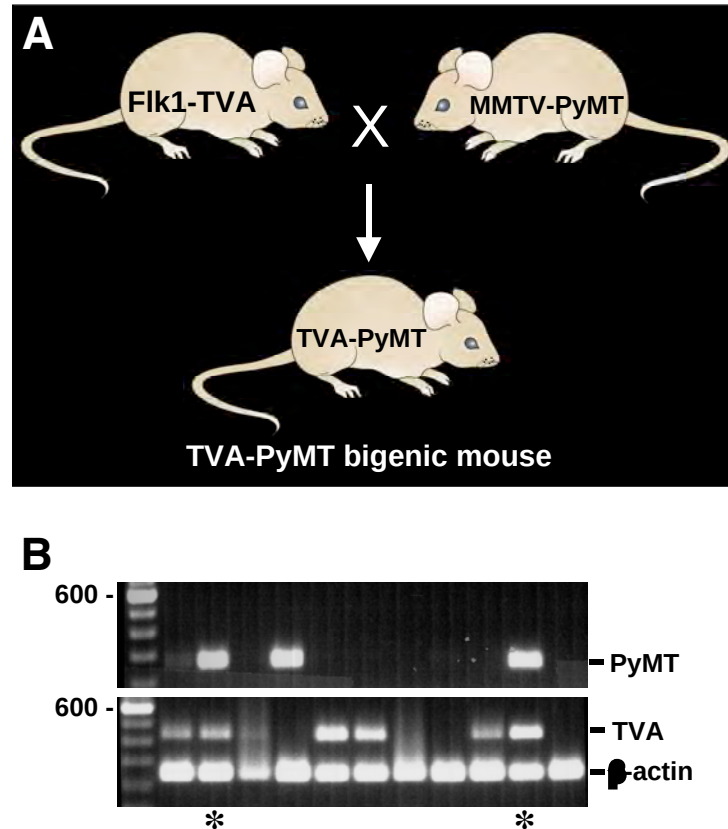


Fig. 7. Flk1-TVA;PyMT bigenic mouse model to study angiogenesis in mammary tumors. (A) Scheme of mouse crosses. Flk1-TVA transgenic mice were crossed with transgenic mice expressing the polyoma middle T (PyMT) oncogene under the control of the mouse mammary tumor virus long terminal repeat (MMTV). Female MMTV-PyMT mice develop multifocal, metastatic mammary adenocarcinomas with short latency and predictable time course. TVA expression in new vasculature of bigenic Flk1 TVA;PyMT mice made blood vessels in the tumors susceptible to infection by the RCAS virus. (B) Bigenic mice were identified by PCR amplification based on the presence of the PyMT oncogene together with the TVA receptor (asterisks).

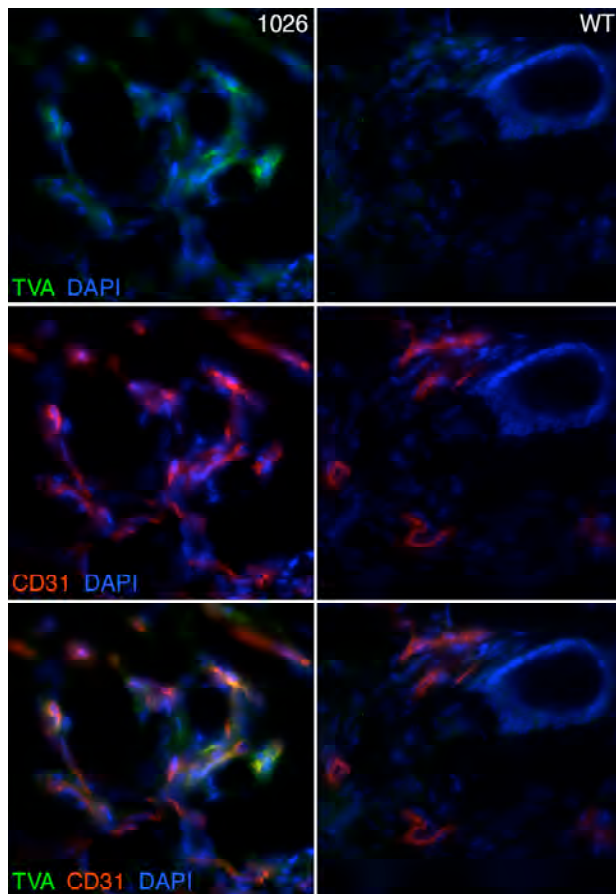


Fig. 8. *In vivo* assay to determine expression of the TVA receptor in tumor endothelial cells of Flk1-TVA;PyMT bigenic mice. Flk1-TVA;PyMT bigenic female mice (from line 1026) and control PyMT mice (WT) were sacrificed at approximately 3 months of age and tumors were removed, frozen and stained with anti-TVA antibodies to detect cells expressing the TVA receptor (green) and with anti-CD31 antibodies to visualize blood vessels (red). All pictures were taken at the same exposure and identically adjusted in Photoshop. Note that a subset of endothelial cells is stained with the TVA antibodies in the 1026;PyMT transgenic line but not in the PyMT line. The fact that some blood vessels do not express TVA suggests that not all blood vessels in these tumors are actively growing.

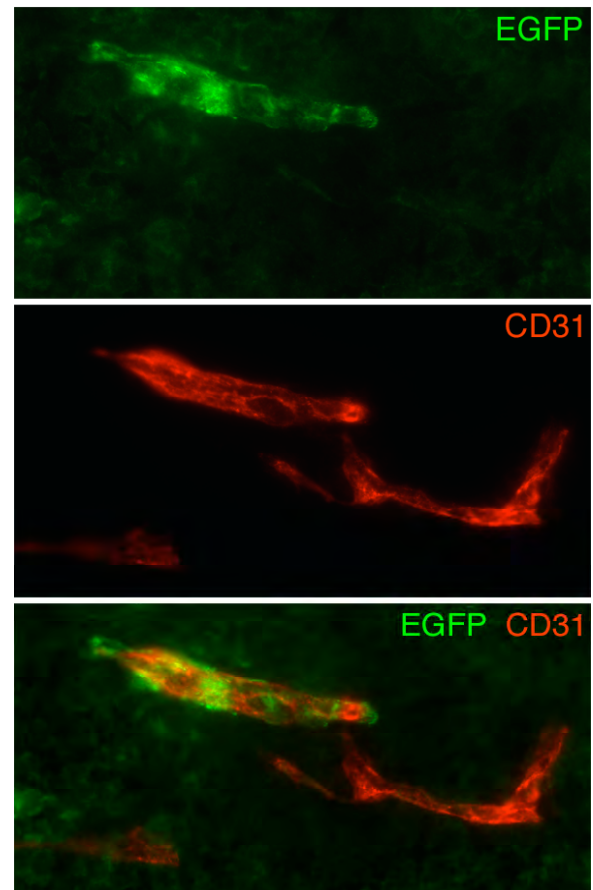


Fig. 9. *In vivo* assay to determine susceptibility of mammary tumor endothelial cells to infection with the RCAS retrovirus. RCAS virus encoding EGFP-PyMT was injected in a vein connected to the tumor vasculature in a 2 month old Flk1-TVA;PyMT bigenic female mouse (from line 1026). The transgenic PyMT expressed in the tumor cells under the control of the MMTV-LTR drives formation of mammary tumors. The PyMT encoded by the RCAS virus served to enhance proliferation of the infected endothelial cells, while the EGFP tag served as a marker to identify the infected cells. The tumors were removed approximately one month after injection of the virus and frozen. Sections were double-labeled with anti-EGFP antibodies (green) and anti-CD31 antibodies to label endothelial cells (red). All pictures were taken at the same exposure and identically adjusted in Photoshop. Note that endothelial cells in only one of the three blood vessels shown are infected.

Key Research Accomplishments

Establishment of TVA transgenic lines

- Constructed a vector containing the cDNA encoding the TVA receptor and the Flk1 promoter and enhancer sequences.
- Injected mouse oocytes with the Flk1-TVA DNA and implanted them in foster mothers.
- Developed a PCR protocol for screening genomic DNA.
- Obtained 17 positive founders.
- Obtained 15 Flk1-TVA transgenic lines (2 of the founders did not transmit the transgene to the progeny).
- Obtained 13 Flk1-TVA transgenic lines that bred consistently (2 lines could not be easily propagated).

Detecting expression of TVA mRNA in transgenic mice

- Developed RT-PCR protocol to quantitate overall TVA mRNA.
- Determined approximate expression levels of TVA mRNA in P3-P5 mouse brains from the different lines. This showed that 12 of the 13 transgenic lines that bred consistently expressed TVA mRNA at different levels.
- Established an *in situ* hybridization protocol to determine the pattern of TVA expression in the different transgenic lines using Flk1 antisense mRNA as a positive control.
- Prepared TVA sense and antisense RNA probes for *in situ* hybridization experiments.
- Prepared Flk1 RNA probes to identify proliferating endothelial cells in the *in situ* hybridization experiments.
- Obtained E10-E11 embryos from all lines and used them for whole mount *in situ* hybridization experiments.
- Identified 6 lines that express TVA in embryonic vasculature with a pattern similar to Flk1.

Detecting expression of TVA protein in transgenic mice

- Constructed vector for expression of GST-TVA protein in bacteria.
- Injected GST-TVA purified from bacteria into rabbits to obtain immune serum.
- Affinity-purified anti-TVA antibodies from immune serum.
- Established immunoblotting and immunohistochemistry protocols to detect TVA protein using TVA-transfected cells.
- Established an *in vivo* Matrigel plug assay with the TVA transgenic mice as an assay to stimulate the growth of new blood vessels in adult mice.
- Used the anti-TVA antibodies to identify the 2 transgenic lines (1026 and 1029) that express the highest levels of TVA receptor in the newly formed blood vessels growing into the Matrigel plugs.

Infecting the endothelial cells of TVA mice with RCAS virus

- Engineered an RCAS viral construct encoding ephrin-B2 fused to EGFP, to be used to infect TVA-positive endothelial cells in Matrigel plugs and tumors.
- Engineered an RCAS viral construct encoding the Polyoma Middle T oncogene fused to EGFP, to be used to infect TVA-positive endothelial cells in Matrigel plugs and tumors.

- Prepared high-titer infectious RCAS viruses encoding EGFP, β -galactosidase, ephrin-B2-EGFP, and EGFP-PyMT for *in vivo* Matrigel and mouse tumor experiments.
- Used the *in vivo* Matrigel plug assay to confirm infectability of proliferating endothelial cells by the RCAS virus.
- Detected clusters of infected (EGFP-positive) endothelial cells in the Matrigel plugs of line 1026, line 1029, and in some instances line 14, when using RCAS-EGFP-PyMT virus.

Crossing TVA transgenic mice with transgenic mice that develop mammary tumors

- Crossed TVA line 1026 with Polyoma Middle T transgenic mice.
- Stained mammary tumor tissue from bigenic Flk1-TVA;PyMT mice with anti-TVA antibodies demonstrating TVA receptor expression in new blood vessels.
- Developed a protocol to inject concentrated RCAS virus in a vein located near the periphery of a tumor and whose ends were closed off to prevent blood flow, thus prolonging exposure of tumor endothelial cells to the virus. This approach will also be generally useful for local delivery of viruses that can infect all mouse cells.
- Verified infectability of proliferating tumor endothelial cells by RCAS-EGFP-PyMT virus. No infected (EGFP-positive) cells were detected when RCAS-EGFP was used, consistent with the proliferative effects of PyMT in endothelial cells.

Reportable Outcomes

- Twelve lines of transgenic mice expressing the TVA receptor mRNA under the control of the Flk1 promoter and enhancer.
- Six lines of transgenic mice expressing TVA receptor mRNA selectively in developing vasculature under the control of the Flk1 promoter and enhancer.
- Two lines expressing TVA protein at high levels in adult new vasculature, including mammary tumor vasculature, and susceptible to infection by the RCAS virus.
- Antibodies to the TVA receptor.
- RCAS retrovirus expressing ephrin-B2-EGFP and an EGFP-Polyoma Middle T fusion protein.
- Funding obtained based on work supported by this award:
NIH grant R01 CA98778 (awarded to Dr. R. Oshima for the period from 1/1/03 to 12/31/07). The third aim in this NIH R01 grant proposes to use the Flk1-TVA transgenic mice generated in the course of this project to target the Ets2 transcription factor in the vasculature of mammary tumors.
- Abstract for DOD BCRP Era of Hope 2005 Meeting (enclosed in the appendix):
“Targeting the mammary tumor vasculature using the TVA-RCAS system”, Vervoort VS, Lu M, Valencia F, Breier G, Pasquale EB.
- Presentations:
“Targeting the mammary tumor vasculature using the TVA-RCAS system”
DOD BCRP Era of Hope 2005 Meeting (June 11 2005).
“Targeting tumor vasculature using the TVA-RCAS system”

Burnham Institute's Signal Transduction Program Meeting (November 7, 2005)

- Manuscript:
Vervoort VS, Lu M, Lesperance J, Seddon JA, Valencia F, Breier G, Oshima R, Pasquale EB. A novel Flk1-TVA transgenic mouse model to study the effects of proteins delivered to adult neovasculature and hematopoietic progenitor cells using the RCAS retrovirus. (in preparation).
- Several review publications acknowledging support from the DOD BCRP program:
Murai KK, Pasquale EB (2004). Eph receptors, ephrins, and synaptic function. **The Neuroscientist** 10:304-314.
Yamaguchi Y, Pasquale EB (2004). Eph receptors in the adult brain. **Curr. Opin. Neurobiol.** 14:288-296.
Ethell IM, Pasquale EB (2005). Molecular mechanisms of dendritic spine development and remodeling. **Progress Neurobiol.** 75:161-205.
Pasquale EB (2005). Eph receptor signaling casts a wide net on cell behaviour. **Nat. Rev. Mol. Cell. Biol.** 6:462-475.
Pasquale EB. Eph receptors and ephrins. In "**Angiogenesis & Vascular Biology: Basic Aspects**". Eds. G. Rubanyi and M Simons, Imperial College Press, Chapter 18, pp. (invited review).

Conclusions

We have developed a new transgenic mouse model that can be used to examine the effects of proteins selectively expressed in the adult neovasculature. In this Flk1-TVA mouse model, newly forming blood vessels selectively express the TVA receptor for the RCAS avian retrovirus, which confers susceptibility to viral infection. This mouse can be used to study the function of proteins delivered using the RCAS virus to angiogenic blood vessels in the adult without also affecting embryonic blood vessels, established blood vessels, and/or cells outside the vasculature. The time of protein expression can be controlled by the timing of virus delivery. In addition, the Flk1-TVA;PyMT bigenic mouse model derived by crossing the Flk1-TVA mouse with the PyMT transgenic mouse breast cancer model, in conjunction with the RCAS gene delivery system, will enable identification of proteins that play a role in mammary tumor angiogenesis. Although we dedicated an additional year and additional resources to this project, we have not yet been able to reach infection levels high enough to conclusively determine the effects of increasing or decreasing ephrin-B2 expression in tumor blood vessels. We anticipate that this will require systemic and repeated delivery of the virus.

The Flk1-TVA transgenic mice developed in this project represent a new animal model ideal for studying proteins that play a role in adult neovascularization, which will facilitate identification of targets for new anti-angiogenic therapies. The growth and metastatic potential of human breast cancer rely greatly on an adequate blood supply. Anti-tumor therapies aimed at disrupting tumor blood vessels in order to starve the tumor cells and thus arrest cancer growth can, therefore, be very effective.

The Flk1-TVA transgenic mouse model joins several other TVA mouse models that have been used in cancer research (Fisher et al., 1999; Wu and Pandolfi, 2001; Jonkers and Berns, 2002; Orsulic, 2002). In particular, another endothelial promoter, that of the Tie2 angiopoietin receptor, has been recently used to drive TVA expression (Montaner et al., 2003; Montaner et al.,

2004; Sodhi et al., 2004). In the Tie2-TVA model, however, all adult endothelial cells express the TVA receptor. This is in contrast to the more selective Flk1-TVA mouse model that we have developed, in which only endothelial cells in newly forming blood vessels express the TVA receptor. In addition, the Flk1-TVA transgenic mice will also be useful for studying the role of proteins delivered to hematopoietic progenitor cells, which are Flk1-positive and give rise to blood cells, endothelial cells and cardiac cells. A manuscript describing the new TVA transgenic model is in preparation, and we will maintain lines 1026 and 1029 and make them available to the research community together with the other resources generated in the course of this project (see below).

Personnel Supported by This Grant

Elena Pasquale, PI	2002-2005*
Mark Lu, Research Technician	2002-2003
Virginie Vervoort, Postdoctoral Associate	2003-2005*

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Appendices

- Abstract for DOD BCRP Era of Hope 2005 Meeting (enclosed in the appendix):
"Targeting the mammary tumor vasculature using the TVA-RCAS system", Vervoort VS, Lu M, Valencia F, Breier G, Pasquale EB.

TARGETING THE MAMMARY TUMOR VASCULATURE USING THE TVA-RCAS SYSTEM

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The growth and metastasis of breast tumors relies on an adequate blood supply, which is ensured by new blood vessels that grow through a process called tumor angiogenesis. Breast tumors of 1-2 millimeters cannot grow further unless they induce the formation of new blood vessels, which also allow the tumor cells to spread to other regions of the body. Many studies have now shown that breast cancer is angiogenesis-dependent, but the molecular mechanisms involved are not completely understood. Blocking tumor angiogenesis is currently one of the most promising experimental cancer treatments, but it is essential to first dissect the molecular events linked to angiogenesis before we can design new treatments.

Ephrin-B2 is a transmembrane protein required for the formation of the embryonic vasculature and is also highly expressed at sites of adult neovascularization, including tumor blood vessels. ephrin-B2 stimulation can induce tumor growth. Thus, interfering with signaling through ephrin-B2 expressed in tumor endothelial cells should inhibit tumor angiogenesis and, as a consequence, breast cancer growth and metastasis. Our goal is to determine if ephrin-B2 represents a viable target for new antiangiogenic therapies to control breast cancer progression.

In order to test this hypothesis, we have designed a new transgenic mouse model that expresses the avian TVA receptor for the RCAS retrovirus under the control of the VEGF receptor 2/Flk1 promoter. Consequently, TVA receptor expression is restricted to the new vasculature, and any cDNA of interest can be selectively delivered to growing blood vessels using the RCAS virus as a delivery agent.

We were able to generate several TVA transgenic mice, which were screened for their TVA mRNA expression level and expression pattern using RT-PCR with early postnatal brain and in situ hybridization in 10.5-day embryos. At least one line expresses high level of TVA mRNA specifically in embryonic blood vessels.

To validate our model and confirm susceptibility of the TVA transgenic mouse line to infection with recombinant RCAS retrovirus, we will use an in vivo matrigel assay. In this assay, endothelial cells will be infected with RCAS retrovirus encoding EGFP as a fluorescent marker and Polyoma Middle T to enhance proliferation of the infected cells.

The effect of ephrin-B2 on neovascularization will then be investigated using this TVA transgenic mouse line, which we have crossed with a transgenic mouse mammary tumor model. This bigenic mouse line, expressing the TVA receptor in mammary tumor

blood vessels, will be infected with a dominant-negative ephrin-B2-EGFP expressing RCAS retrovirus, which we already have successfully produced.

The TVA transgenic mouse model that we have generated will also be a useful tool for studying the effects of other proteins of interest expressed in tumor endothelium using the RCAS retrovirus system.

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