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**Principal Investigator:** Lee (*Last Name*) William (*First Name*) M. F. (*MI*)

**Proposal Title:** Targeting Tie2 to Increase Breast Cancer Responsiveness to Antiangiogenic Therapy

**Introduction**

Antiangiogenic therapy of cancers involves inhibiting tumor blood vessel development to deprive tumors of vital oxygen and nutrients. The potential benefits of antiangiogenic strategies have been dramatically shown in mouse tumor models. Results in human clinical trials, however, have been less striking. Recent trials have shown survival benefits, but tumor regression, which is often reported in murine tumors, is rarely seen in treated human cancers. A potential explanation for this disparity in treatment outcomes is that the vasculature of human tumors may be more resistant to antiangiogenic therapies. This may be due, at least in part, to extensive pericyte coverage of vessels in many common human cancers, such as breast cancers, compared to a relative paucity of pericytes surrounding vessels in commonly studied mouse tumors. Pericytes are periendothelial mesenchymal cells that surround capillaries, are thought to be a marker of vessel maturity and stability, and may confer resistance to certain antiangiogenic agents. Mouse mammary tumor virus (MMTV)-induced mammary carcinomas arising in C3H/HeN mice may more faithfully model human breast cancer vasculature inasmuch as vessels in these tumors have extensive pericyte coverage like in human breast cancers. Interestingly, we found these tumors were resistant to rIL-12 antiangiogenic therapy, which was effective against every other mouse tumor model tested.

The endothelial-specific receptor tyrosine kinase, Tie2, regulates microvessel pericyte coverage. Studies in this IDEA award will examine whether inhibiting Tie2 activation diminishes tumor vessel pericyte coverage in transplanted mouse tumors and MMTV mammary tumors and enhance their susceptibility to antiangiogenic therapy. We will first develop an inducible system for inhibiting Tie2 activation using the extracellular domain of Tie2 (Tie2Ex) as a decoy receptor in K1735 melanoma cells. These are easily transfectable tumor cells and produce tumors with well-characterized vasculature. Once the inducible system has been validated in this system, it will be brought into MMTV-induced mammary carcinomas using transgenic manipulation of mice.

**Body**

*Task 1.* Determine whether blocking Tie2 reverses pericyte coverage in K1735 tumors.

a. Develop K1735 cell line that inducibly expresses Tie2Ex (Months 0-4)

We chose the tetracycline (doxycycline)-inducible ("Tet-On") system for regulating Tie2Ex expression in tumors. Tie2Ex is the soluble, extracellular domain of the Tie2 receptor, a decoy receptor for Tie2 ligands and an inhibitor of Tie2 activation. To achieve its regulable expression in tumors, we first transfected K1735 tumor cells to constitutively express the reverse tetracycline transactivator (rtTA) , which activates transcription of genes under the control of a tetracycline-response element (TRE) only in the presence of doxycycline. A plasmid (pEF2-rtTA) containing rtTA under the control of the eukaryotic translation initiation factor 1-alpha (eIF-1a) promoter and a neomycin-resistance gene was used for transfection. A single clone of transfected cells, K1735.m39, was chosen for subsequent study because these cells showed little expression of luciferase from a transiently transfected TRE-luciferase gene in the absence of Dox and abundant expression of luciferase in the presence of Dox. K1735.m39 cells were then transfected with a plasmid containing the TRE-Tie2Ex gene plus a hygromycin-resistance gene or the empty, hygromycin-resistance plasmid. We selected a clone of transfected cells, K1735.Tie2Ex$^{ind}$ cells, that secreted almost no Tie2Ex when there was no Dox in the medium and that secreted abundant Tie2Ex when 1 μM Dox was added. The corresponding control K1735.m39hygr$^{r}$ cells did not express Tie2Ex with or without Dox. The K1735.Tie2Ex$^{ind}$ cells secreted intermediate levels of Tie2Ex when lower concentrations of Dox was added and shut off Tie2Ex expression when Dox was removed.

These studies were delayed by >6 months due to problems with the poor performance of the CMV-rtTA plasmid we obtained from BD Bioscience. This plasmid gave very weak induction of TRE promoters in transfected K1735 cells. Analysis of the problem revealed there was weak rtTA expression in the transfected cells due to (a) CMV being a relatively weak promoter/enhancer in K1735 cells and (b) a poor translation initiation [Kozak] consensus site in the rtTA mRNA. To solve these problems, we changed

the rtTA plasmid to pEF2 and mutated its translation initiation site to a canonical Kozak consensus sequence. Discovery, analysis and resolution of the problem took about 8 months.

b. Determine if Tie2Ex reverses pericyte coverage of vessels in K1735 tumors (Months 5-8).

To study the in vivo operating characteristics of our Dox-inducible system, we injected C3H/HeN mice with K1735.Tie2Ex$^{ind}$ cells and control K1735.m39hygr$^{r}$ cells. These cells formed tumors with slower growth kinetics than wild-type K1735 cells. When tumors reached 6-8 mm diameter, Dox (1 mg/ml) was placed in the drinking water of some of the tumor-bearing mice. After two days, the mice were euthanized and tumors removed for protein analysis. Western blot analysis of tumor lysate showed that K1735.Tie2Ex$^{ind}$ tumors from mice given Dox in their drinking water had easily detectable Tie2Ex, whereas tumors from mice not given Dox had no Tie2Ex, and K1735.m39hygro$^{r}$ tumors had no Tie2Ex whether or not their host was given Dox (see Figure) . Thus, tumors created from K1735.Tie2Ex$^{ind}$ cells showed expected Dox-inducible expression of Tie2Ex. We are in the process of studying whether tumors with induced Tie2Ex expression have altered pericyte coverage of their vessels. Briefly, mice bearing K1735.Tie2Ex$^{ind}$ or K1735.m39hygro$^{r}$ tumors were given Dox (or not) in their drinking water for 2 days, 7 days and 14 days prior to tumor harvesting. Protein lysates were prepared from a portion of the tumor and the remainder were processed for histological analysis. These studies will be finished in the next month.

*Task 2.* Determine whether Tie2Ex blocks or reverses pericyte coverage of vessels in MMTV-induced breast tumors.
a. Develop transgenic mice that inducibly express Tie2Ex (Months 5-16).
b. Cross mice that inducibly express Tie2Ex with MMTV infected C3H mice (Months 17-24).
c. Determine if transgenically expressed Tie2Ex blocks or reverses pericyte coverage of vessels in MMTV-induced breast tumors (Months 24-36).

We have excised and purified the TRE-Tie2Ex transcription unit from its plasmid, and given the DNA to the transgenic mouse facility of the University of Pennsylvania. The will soon inject this DNA into fertilized eggs from C3H/HeN mice and implant the injected eggs into pseudopregnant female. Pups born from these females will be screened for presence of TRE-Tie2Ex in their DNA. An investigator at the University of Pennsylvania, Dr. Lewis Chodosh, has FVB mice that express rtTA in mammary glands due to a MMTV-rtTA transgene that he introduced into their germline. He has agreed to give us these mice in a collaborative study. The TRE-Tie2Ex transgenic mice will be with his MMTV-rtTA mice to obtain MMTV-rtTA x TRE-Tie2Ex double transgenics that should express Tie2Ex in mammary tissue and their tumors when Dox is placed in their drinking water. MMTV-induced mammary carcinomas arising in these mice should have Dox-inducible expression of Tie2Ex and allow us to study the effect of Tie2Ex on pericyte coverage of vessels in these tumors.

*Task 3.* Determine if Tie2Ex increases responsiveness of MMTV-induced breast tumors to antiangiogenic therapy (Months 24-36).

This Task will naturally proceed once Task 2 is accomplished.

**Key Research Accomplishments**

Creation of K1735.m39 cells that allow induction of genes under the control of TRE promoters.
Creation of K1735.Tie2Ex$^{ind}$ cells that allow Dox-inducible expression of Tie2Ex in vitro and in vivo.

**Reportable Outcomes**

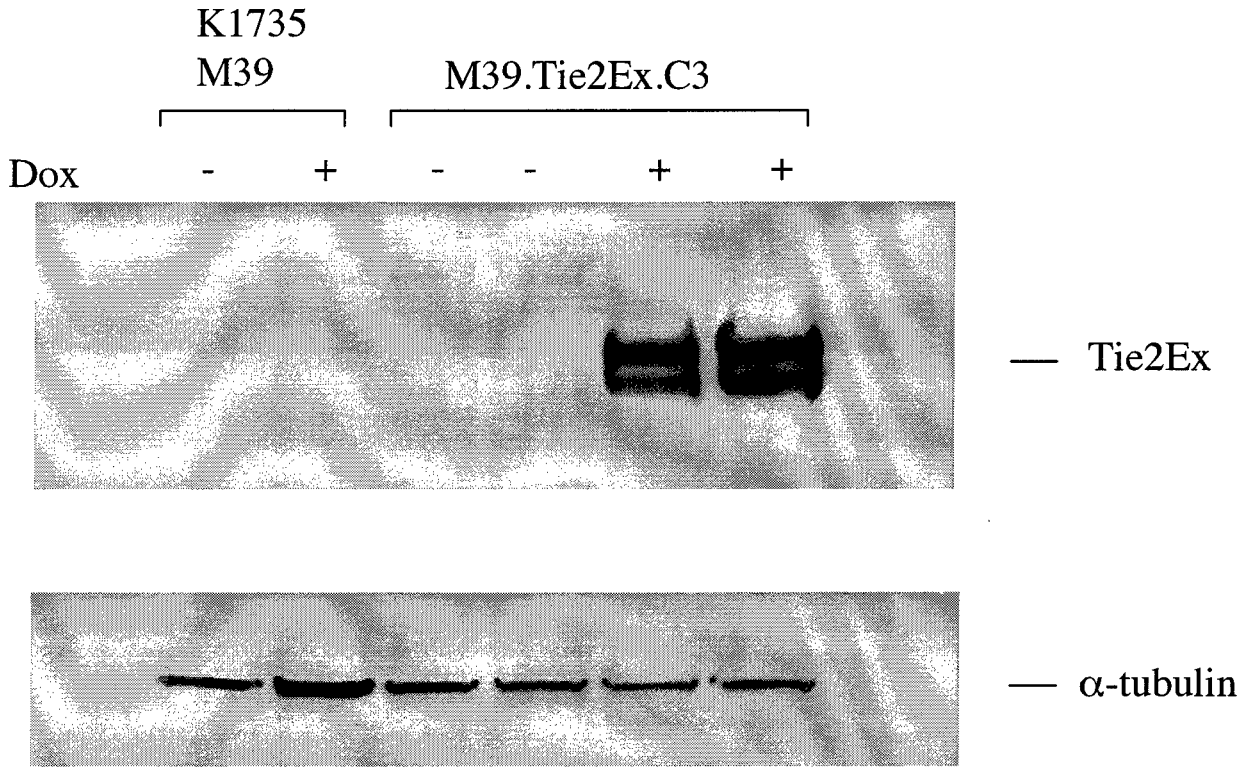
None

**Conclusions**

Inducible expression of Tie2Ex in mammary carcinomas should be achieved using the system chosen.

**References and Appendices**

Presentation P19-9 at Era of Hope meeting, Philadelphia, PA (6/8/05 - 6/11/05).


K1735
M39
M39.Tie2Ex.C3
Dox
-
+
-
-
+
+
Tie2Ex
α-tubulin