The Columbia University Surgical Resident’s Guide to Securing NIH LRP Funding
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Outline

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Brief Overview of the NIH LRP Program

Despite sincere interest in research, many physicians strapped with significant medical school debt cannot afford to take time away from the clinical practices which support their salaries. Recognizing that these forces would ultimately lead to a critical shortage of research talent, the NIH established the Loan Repayment Program (LRP) in an effort to encourage young, motivated physicians to pursue their research efforts by offering to directly pay-off a portion of their student loan debt. By reducing medical school loans, the NIH is hoping that doctors will be able to devote more of their careers to research time without feeling financially obligated to engage only in clinical activity.

This background information is important, as the “mission” of the LRP is something that should be reflected in your personal statement and research proposal, described later on.

Nearly all the information about the NIH LRP can be found at their website, which is extremely thorough and readily navigable:
http://www.lrp.nih.gov

The LRP is a composite of 5 different programs, and step 1 is to decide which program you will best qualify for:

1) Clinical Research LRP ➔ patient-oriented research conducted with human subjects or materials (i.e., tissue specimens); includes outcomes, clinical trials, new technologies, etc.
2) Pediatric Research LRP ➔ directly related to diseases, disorders, and other conditions in children.
3) Contraception & Infertility Research LRP ➔ research whose long-range objective is to ameliorate the failure of couples to conceive or provide methods of preventing pregnancy.
4) Health Disparities Research LRP ➔ basic, clinical, or behavioral research directly relevant to health disparity populations and the medically underserved.
5) Clinical Research LRP for Individuals from Disadvantaged Backgrounds ➔ clinical research, as above, for applicants from a low family income level background.

Applications should be filed as EXTRAMURAL NEW APPLICATIONS. The intramural is for NIH employees.

Pre-Application Checklist

Define your research commitment
The LRP requires only 20 hours per week of dedicated research time. While this is easily accomplished during protected research years, it implies that residents may even be eligible for LRP funding during their clinical years if they can document the 20 hours of weekly research time. You should establish a research commitment with a laboratory and PI no later than 9–12 months before you begin research. Early planning is key to maximizing your chance of submitting a fundable application.

Carefully review eligibility requirements on the website (http://www.lrp.nih.gov/about/eligibility.htm)
While most U.S. surgical residents meet the basic eligibility requirements, applicants should carefully review the eligibility requirements on the website, paying particular attention to citizenship requirements, qualifying “repayable” debt, and any conflicting pre-existing service agreements with the NIH or other federal agencies (i.e., NRSA, military, etc).

Identify a specific research project that is receiving current funding
Only funded research can qualify for the LRP. Fortunately, the LRP definition of “funded research” is very broad, and so even Department of Surgery funding and small private grants can validate a research project for LRP eligibility, as well as projects which receive more robust NIH funding (R01, K08, etc.). Again, LRP funds are forwarded directly to your loan lenders, and are not meant to support your salary or research costs. Thus, your project must have its own independent sources of funding.
Select the applicable LRP program based on your research project

LRP applications are submitted to one of the five component programs listed above, into which most projects can be structured. Even the most basic science research should have clinical implications which can qualify it for the Clinical Research program.

Since the application precedes your actual research time by as much as 1 year, the specifics of your research proposal may differ from the exact project in which you engage, as lab efforts can be redirected in the interval between your application and your start date. Use this to your advantage. That is, choose the project in your lab which has the greatest potential, most secure funding, and/or best-fits into one of the 5 LRP categories. Even if you ultimately engage in a different project, the NIH understands the dynamic nature of research, and you are not required to stick to every hypothesis and research experiment proposed in your application.

Actively engage your Research Advisor (i.e., Principal Investigator, P.I.) in your LRP application efforts

Ultimately, it is the Research Advisor’s credibility, funding, and ability to provide a productive research environment which will help the NIH decide whether to fund your efforts. You must engage your Research Advisor early in this process, so that they can assist you in drafting a credible research proposal. It is unwise to attempt to draft a research proposal based only on a brief meeting with the P.I. and reading previous lab publications. The Research Advisor should provide you with previous grant proposals they have written and have time to review your application before submission.

Verify all eligible loans and collect relevant documents

The LRP will require detailed information about each “eligible loan” (see website for loan eligibility – most loans are eligible, federal and private). They will want to see a copy of the original promissory note and original loan amount, as well as current information about interest rates, remaining balances, active payments, capitalized interest, deferments, consolidations, penalties, etc. Most of this can be obtained off each lender’s website; however, promissory notes may need to be sent by mail.

Timeline for Application Process

deadlines are subject to change; verify important dates on the LRP website

Year Prior to Research Start Date

- **July** → **Choose research lab**: interview various labs and P.I.’s to identify the best match.
- **August** → **Pre-application checklist**: review above with P.I., gather loan documents, review LRP website
- **Sept 1** → **Extramural application cycle begins**: create account on LRP site; fill out demographic info.
- **Sept 15** → **Review application specifics**: word-length limits and content requirements for each component.
- **Oct 1** → **Enter Loan Information**: begin filling out this section with all relevant info on eligible loans.
- **Oct 1** → **Complete Online Certifications**: application Step 1; forms may need to be signed and faxed
- **Oct 15** → **Identify Recommenders**: contact potential recommenders and enter their info on the website.
- **Oct 15** → **Engage Institutional Contact**: email contact directly, prior to generating the website auto email
- **Nov 1** → **Complete drafts of Research Activities & Personal Statement**: review these with P.I.
- **Nov 15** → **Final CV (Biosketch) Update**: include all previous research experience and publications.
- **Dec 1** → **Extramural application cycle closes**: all application materials must be submitted by this date.

Year Research Will Begin

- **Jan 9** → **Support Materials Due**: P.I., recommenders, and institutional contact must meet this deadline
- **Jan-Mar** → **LRP Contract, Loan Materials**: additional documents requested by NIH; check email regularly
- **July 1** → **Begin Research**: residents without protected time should dutifully keep track of research hours.
- **Aug 1** → **LRP Funding Decision Announced**: funding amount and “participant obligations” to loan repayment announced.
Application Components

Below is the standard application screen of the LRP web interface. This is the screenshot of a RENEWAL application, and therefore differs slightly from a NEW application, most specifically in that new applications will have an additional section for entry of your medical school loan information.

The general design of the application interface requires you complete various sections, and then formally “SUBMIT” each section as it is completed. Realize that once a section is submitted, it cannot be modified. Sections can be partially filled out: click the “Continue” button at the bottom of each section and information will be saved.

Step 1: Personal Information

- **Applicant Information** → general demographics. Indicate your *Position Title* as ‘Postdoctoral Fellow’. Any pre-existing NIH service obligations must be listed in this section as well. There is a section to upload your *Biosketch (C.V.)* – you can wait to do this until just before you are ready to submit the completed application, as you want the most up to date information reflected in the Biosketch. A link to instructions on how to format the Biosketch and sample biosketches is provided: ([https://lrp2.od.nih.gov/NIHLRPApplication/biosketch/biosketch_help1.htm](https://lrp2.od.nih.gov/NIHLRPApplication/biosketch/biosketch_help1.htm))

- **Funding Information** → information about the funding which supports the research laboratory you will be participating in. There are separate sections for non-profit funding sources, private funding sources, and NIH support. This is not where you enter information about your specific loans.

- **Personal Statement** → can be uploaded as a Word document, limited to 8000 characters (with spaces) which is about 2 single-spaced typed pages. As usual, shorter is better. It is very important to address the points which the LRP are interested in:
  a) Previous Research Training
  b) Short term academic and research objectives
  c) Long term career objectives, and plans to achieve these objectives

Review the sample Personal Statement at the end of this guide. Be sure to include appropriate page-headers. Review the Background Information on the LRP written above – the general message you want to convey is that you are someone with potential to become a successful researcher, and that the lab you are entering will provide the necessary training to make you an active investigator (short-term goals). By helping repay your med school debt, the NIH can improve your chances of engaging in an active research career (i.e., you will justify their investment). Also describe your plans for future fellowship training (long term career objectives), and how this too will enhance your growth as a surgeon-scientist (whose access to research time would be enhanced by loan repayment). Avoid sob-stories about financial hardship or excessive information about personal “attributes”; focus more on the tangibles of research training and how you will use it to guide your career.

- **Certifications** → forms that should be printed, signed, and faxed back to the NIH.
Complying with a Funded LRP Contract

All LRP communication is performed by email, so be sure to check it regularly. The two major compliance points for a funded contract, in addition to any initial paperwork you are asked to complete are: (1) Documenting Research Hours, and (2) Verifying Loan Payments.

Documenting Research Hours ➔ use the E-value system to keep track of research hours during your protected research years. This helps maintain Dept. of Surgery work hours compliance, and also provides a record of your work should it ever be interrogated by the NIH (extremely unlikely). This is most critical for those not taking protected research time. If you apply as a clinical resident, you must document at least 20 hours/wk of research work hours, and be wary of how this affects ACGME work hours. Jessica Kandel has established an online research hours log specifically for the LRP participants, and if you prefer not to enter research hours in E-value (in order to avoid work-hours issues), then the research hours should be documented separately on her site: http://cait.cpmc.columbia.edu:88/dept/surgery/peds/LRP.html
Verifying Loan Payments → the NIH distributes loan payments quarterly to your approved loans – this is usually done electronically. However, after each payment is received by your lender(s), you must fax confirmation that the payment was appropriately applied to your account(s). This information can usually be printed directly from your lender’s website.

A Word About Taxes…

Since loan payments are considered income (basically, a salary from the federal government), the loan payments are subject to income tax… however, not to worry, as the LRP will actually pay the taxes on your payments for you!!

For example, let’s say you are approved for a $35,000 annual LRP loan payment. The NIH actually reports to the IRS that you were awarded closer to $55,000, and it forwards the appropriate amount of income tax on $55,000 (about $20,000) directly to the IRS – so while you never see that money, it effectively makes it so that your loan payments are “tax-free” – you will receive 1099G tax forms at the end of each fiscal year which document both the LRP loan payments made to your lenders and the taxes paid on your behalf by the NIH.

Renewal LRP Applications

The funding rate for 1st time new extramural LRP applications is around 40%, while the funding rate for renewal applications is >70%, and is therefore well worth the consideration of applying. Given that most surgical residents only take 2 years of protected research, you must clearly outline in your renewal application how you plan to comply with the 20hrs/week of dedicated research time. Specifically state how you will log your research hours and that they will be reviewed by your research advisor (E-Value, Kandel LRP site)

The renewal LRP application is almost identical to the new LRP application, with some minor differences:

1) No Loan Details Required → a huge time-saver

2) Addition Section – “Research Accomplishments” → in addition to the personal statement and research activities sections, this section is meant to describe what you’ve accomplished in the previous 2 years of research, and how this will lead you towards additional years of successful work. Include information on talks, posters, publications, and even some diagrams to add depth to this section. It is brief – only about 2 pages.

3) Recommenders → this time the emphasis of your references should be on how productive your 1st 2 years were, and how additional years of research will continue to help you meet your short and long term goals (as outlined in your personal statement).

A sample renewal application is included at the end of this guide.

Sample Application Materials Comprise the Remainder of this Guide

APPLICATION #1: New Extramural Application for Pediatric LRP
“Acquired Resistance to VEGF Blockade in Wilms Tumor”
Jason C. Fisher, MD
-- Personal Statement – pages 6-7
-- Biosketch – pages 8-9
-- Research Activities – pages 10-14
-- Research Environment (submitted by Research Advisor) – page 15
-- Current Funding Sources – pages 16-17

APPLICATION #2: Renewal Extramural Application for Pediatric LRP
“Combined Cyclooxygenase-2 and VEGF Blockade in Wilms Tumor”
Jason C. Fisher, MD
-- Personal Statement – pages 18-19
-- Biosketch – pages 20-22
-- Research Accomplishments – pages 23-24
-- Research Activities – pages 25-29
-- Current Funding Sources – pages 30-31
I began surgical training with a firm work ethic and a dedication to children, and have nourished my clinical passions alongside an evolving research portfolio. Surgeons combine technical training with clinical medicine. This combination may offer particular advantages, as awareness of clinical relevance is combined with the ability to perform technically challenging experiments. I will strive to become a true physician-scientist as I embark on two years of protected research in the area of treatment-refractory pediatric cancer.

Previous Research Training

I greatly admire physicians who resist the pressure to specialize early, and can train to become great generalists before evolving into great specialists. Inspired by my colleagues, I have sought to mold my research experiences accordingly. I have participated in diverse areas of scientific study: basic science, clinical research, outcomes assessments, and computational biologic modeling. Familiar now with numerous avenues of research, I can draw on these experiences to guide specific research initiatives that will form the core of my career development.

Early in medical school I met Dr. Patricia K. Donahoe, who invited me to join her research efforts in the Department of Pediatric Surgery at the Massachusetts General Hospital. She introduced me to Dr. Jay J. Schnitzer, who would become my first research mentor. I was quickly hooked on the thrill of predicted data coming to bear in the lab, and worked summers and after-hours in tandem with my medical school curriculum. Funded by an NIH-HL grant, my work focused on congenital diaphragmatic hernia (CDH), a disease with significant mortality due to lung hypoplasia. My role was to determine the effect of oxidation-reduction on fetal lung growth in the context of CDH. Using a rodent model of CDH, I showed improved lung growth in fetal lung organ culture enriched with N-acetyl cysteine, and correlated this growth with a reduced intracellular redox state. I introduced innovations to our lab by developing new techniques to digitally analyze experimental lungs, and pioneered new use for an enzyme assay in the analysis of redox potential. These techniques and experiments were published and presented nationally, and received the top prize in the National Medical Student Research Award competition sponsored by the Association for Academic Surgery.

I complemented basic science work with clinical projects at the Boston University School of Medicine. I collaborated with Dr. Paul Schroy of the Department of Gastroenterology to address the deficiency in familial colorectal cancer (CRC) risk assessment which exists among primary care providers. Funded by an NCI grant, I created software for PDA devices to assist housestaff in determining CRC risk. This program was used in a randomized clinical trial where it provided a significantly improved knowledge of familial CRC among clinicians.

I began surgical residency at Columbia University in 2003. Dr. Mark A. Hardy (Program Director) and the surgical faculty have nurtured my clinical judgment and technical skills as I train to become a highly skilled academic surgeon. With focus still on childhood disease, I began a project with Dr. Larry Bodenstein of the Department of Pediatric Surgery, and secured two Startup Grant Awards from Columbia for our efforts. Drawing on my past work on congenital diaphragmatic hernia, we examined diaphragm embryology and anomalies driving CDH. We developed a novel cell-based, computer model to evaluate various theories of diaphragm growth. Our model used data collected by imaging serial sections of mouse embryos. The simulations we generated suggested mechanistic flaws in more recent theories on diaphragm growth. These results are accepted for publication, and will be presented at the 2006 Children’s National Medical Center annual Symposium on Extracorporeal Life Support. Keeping continued focus on the care of children, I assessed outcome data on all pediatric thyroid cancers treated at Columbia over the past 15 years. With Dr. Kathryn Spanknebel of the Department of Surgery, we compiled data on 47 children with thyroid cancer, a rare disease in children, but plagued by high recurrence rates. We used Kaplan-Meier and Cox regression analysis to identify risk factors associated with disease recurrence, and presented these data at the 2005 New York Surgical Society Scientific Session.
Short-Term Research Objectives

Regarding my short-term research objectives, I plan to:
1. Acquire new skills in molecular biology, genetic analysis, and in-vivo tumor modeling to achieve proficiency as a basic science researcher.
2. Learn to critically evaluate the data generated by experiments that employ these techniques.
3. Become adept at proposing logical, hypothesis-driven approaches to unravel the molecular mechanisms driving the acquired resistance to VEGF blockade in Wilms tumor.

I have been accepted for a two-year fellowship in the Pediatric Tumor Biology Laboratory directed by Dr. Jessica J. Kandel (Pediatric Surgery) and Dr. Darrell Yamashiro (Pediatric Oncology) in the Institute for Cancer Genetics at Columbia University. To combat treatment-refractory cancers, I will study acquired resistance to VEGF blockade in Wilms tumor, using a validated xenograft animal model that permits analysis of vascular adaptation to VEGF blockade. Specifically, I plan to determine the impact of her2/neu expression on tumor angiogenesis, a paradigm for the interaction of oncogenes and VEGF-driven angiogenesis, as approximately one-third of pediatric Wilms tumors are her2/neu(+). The results of these experiments may provide a rationale for use of anti-angiogenic therapies in Wilms tumor, and assist in circumventing their limitations.

Long-Term Career Goals

My long-term career goals involve:
1. Becoming a capable research scientist, contributing to the study of treatment-refractory childhood diseases.
2. Augmenting my clinical and technical skills by seeking advanced training in pediatric surgery.
3. Emerging from training as a competent and motivated physician-scientist eligible for further research funding, and ultimately transitioning into a fully independent R01-funded investigator.

These ambitions start with two years of research with Dr. Kandel beginning July 2006. My surgical training resumes thereafter, completing general surgery residency in July 2010 with plans to then apply for advanced training in pediatric surgery. Of Dr. Kandel’s previously-mentored 11 postdoctoral surgical research fellows, all have gone on to advanced academic training, including 7 training specifically in pediatric surgery. The most senior of these fellows (Dr. Jianzhong Huang) was recently awarded a K08 while being mentored by Dr. Kandel. My work with Dr. Kandel is thereby supportive of both my short-term research goals of battling childhood cancer and my long-term career goals of becoming a pediatric surgeon. The experience I gain these next two years will enable me to continue pediatric research endeavors throughout my career.

My parents are public school teachers for New York City and have always struggled financially. I worked diligently to merit a full-tuition undergraduate scholarship to Boston University, but I incurred great debt in financing my medical education. I remain firm in my career goals as an academic surgeon-scientist, but cannot ignore the crippling burden of my medical school debts. The LRP grant from the NIH is as essential to my career development goals as is my basic science training. With this support, I can continue to work with passion and diligence towards battling childhood disease. I ask only for the opportunity.

References

NAME

Jason C. Fisher, M.D.

POSITION TITLE

General Surgery Resident, PGY-3

EDUCATION/TRAINING  (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

<table>
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<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
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<td>Boston University, Boston, MA</td>
<td>B.A.</td>
<td>1996 – 1999</td>
<td>Medical Science, History</td>
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<tr>
<td>Boston University School of Medicine, Boston, MA</td>
<td>M.D.</td>
<td>1999 – 2003</td>
<td>Medicine</td>
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A. Positions

2003 – Present: General Surgery Residency, Columbia University College of Physicians and Surgeons, NY, NY

Professional Memberships

1999: Current Member, American Medical Association
1999: Current Member, Massachusetts Medical Society
1999: Current Member, American Medical Students’ Association
2002: Current Member, Alpha Omega Alpha
2003: Current Member, New York State Medical Society
2003: Resident Member, Association for Academic Surgery
2004: Resident Member, American College of Surgeons

Honors

1996: Trustee Scholarship – Merit scholarship for full undergraduate tuition, Boston University
2001: Dean’s Accolade for Exceptional 2nd Year Performance, Boston University School of Medicine
2002: Honor Society Inductee, National Association of Pathology Chairs
2002: Honor Society Inductee, Alpha Omega Alpha
2003: Mary Stafford Award for Excellence in Surgery, Boston University Department of Surgery
2003: Top Prize, National Medical Student Research Award, Association for Academic Surgery
2003: Startup Grant Research Award, Columbia University Department of Surgery
2004: Startup Grant Research Award, Columbia University Department of Surgery

B. Peer-Reviewed Publications


National Meeting Oral Presentations


C. Research Support and Previous Experience

*Principal Investigator: Jessica J. Kandel, MD – Department of Pediatric Surgery, Columbia University
Project Status / Role: Awaiting to Participate – Resident Research Fellow
Research Goals: To determine the impact of her2/neu expression on angiogenesis in Wilms tumor
Project Support: Pediatric Cancer Research Foundation – Annual Grant, 2005
NIH R01 Grant – R01-CA100451

Principal Investigator: Lawrence Bodenstein, MD, PhD – Department of Pediatric Surgery, Columbia University
Project Status / Role: Currently Participating – Resident Investigator
Research Goals: Modeling abnormal morphogenesis of the fetal diaphragm with novel computer simulation
Project Support: Surgical Research Startup Grant Award – Department of Surgery, Columbia University

Principal Investigator: Kathryn Spanknebel, MD – Department of Surgery, Columbia University
Project Status / Role: Completed Project – Resident Investigator
Research Goals: Compiled and analyzed outcome data for pediatric thyroid disease
Project Support: New York Thyroid Center, Department of Surgery, Columbia University

Principal Investigator: Jay J. Schnitzer, MD, PhD – Department of Pediatric Surgery, Mass General Hospital
Project Status / Role: Completed Project – Medical Student Research Associate
Research Goals: Identified redox as a key regulator of fetal lung growth in congenital diaphragmatic hernia
Project Support: NIH HL Grant – HL-03132

Principal Investigator: James M. Becker, MD – Department of Surgery, Boston University School of Medicine
Project Status / Role: Completed Project – Medical Student Research Associate
Research Goals: Examined the role of oxidative stress within a mucosa tissue model of ulcerative colitis
Project Support: Department of Surgery, Boston University School of Medicine

Principal Investigator: Paul C. Schroy, MD, MPH – Department of Gastroenterology, Boston University
Project Status / Role: Completed Project – Medical Student Research Associate
Research Goals: Designed software for PDA devices to assist in assessing colorectal cancer risk
Project Support: NCI Grant – K22-CA90680
SUMMARY

While most children with Wilms tumor (WT) are cured, a subset continues to fail all current therapies. In this proposal, we will study the response to blockade of vascular endothelial growth factor (VEGF), a validated new clinical strategy, using a xenograft model of WT. We will focus on changes in endothelium, perivascular cells, and expression of angiogenic genes (VEGF, angiopoietins, platelet-derived growth factor-B (PDGF-B), and Eph/Ephrins). Our pilot data suggests that VEGF blockade initially inhibits WT xenograft growth, yet prolonged treatment leads to tumor recurrence associated with profound alterations in vasculature. We hypothesize that a potential mechanism of this resistance is the remodeling of tumor vessels to a state where they are less dependent on VEGF. We will examine this possibility by studying the her2/neu oncogene, which may contribute to WT response to VEGF blockade. We will determine whether her2/neu expression influences survival during VEGF blockade, by comparing the effects of this blockade in xenografts expressing different levels of her2/neu. We will characterize the effects of her2/neu activation/blockade on angiogenic genes in vitro and angiogenesis in vivo, and determine whether her2/neu inhibition affects acquisition of resistance to VEGF antagonists. The resulting preclinical data may provide a rational basis for use of anti-angiogenic therapies in WT, and assist in circumventing their limitations.

BACKGROUND AND SIGNIFICANCE

Wilms tumor (WT) is the second most common primary abdominal tumor in children. While most children are cured with current regimens, 10-15% develop treatment-refractory and ultimately fatal disease. These patients urgently require new therapies, and might benefit from novel strategies. No single genetic marker for poor outcome in WT has been defined, although increased frequencies of certain molecular alterations are detected in high-risk tumors (e.g. loss of heterozygosity\(^1\), p53 dysfunction\(^2\)). Thus, these cancers are likely to be genetically heterogeneous. We hypothesize that the capacity to develop vasculature with particular features that support tumor growth may be an underlying common theme in aggressive disease.

Acquiring the capacity to induce a new blood supply is a crucial step in tumor development. New blood vessel growth depends on the expression of pro-angiogenic cytokines. VEGF, the best understood, promotes endothelial cell (EC) proliferation and survival, and is nearly ubiquitous in human tumors\(^3\)\(^-\)\(^6\). Regulation of VEGF expression is complex. Upregulation is a physiologic response to hypoxia, mediated by hypoxia-inducible factor 1\(\alpha\) (HIF-1\(\alpha\))\(^7\). However, both HIF-1\(\alpha\) and VEGF are also upregulated by hypoxia-independent oncogenic pathways (e.g. her2/neu\(^8\)\(^-\)\(^13\)).

VEGF blockade has recently been validated as an antiangiogenic strategy in clinical trials\(^14\)\(^-\)\(^16\). For unclear reasons, the efficacy of this strategy varies between tumor types. Previous studies indicate that prolonged hypoxia may cause development of relatively VEGF-independent vasculature\(^17\). Consistent with this possibility, our preliminary in vivo experiments revealed specific alterations in vessel architecture in VEGF-blocked WTs. Thus, we hypothesize that vascular remodeling in WT may: (1) result from chronic hypoxia caused by VEGF blockade; (2) reflect specific changes in expression of angiogenic cytokines; and (3) reflect the influence of oncogenic pathways, such as her2/neu. Dissection of these elements may provide novel data about the processes governing vascular integrity in WT during VEGF blockade.

Misexpression of human epidermal growth factor receptor (HER) family members is implicated in loss of growth inhibition, a key early step in tumorigenesis. Recently, her2/neu overexpression has been detected in a significant proportion of WTs\(^18\). HER family members are linked to the regulation of angiogenesis. In particular, her2/neu is known to regulate both VEGF (via HIF-1\(\alpha\)) and Ang-2 expression in cultured breast cancer cells\(^9\)\(^,\)\(^19\)\(^,\)\(^20\). There is evidence that her2/neu also promotes resistance to apoptosis in
tumor cells\textsuperscript{21,22}. Taken together, these data suggest that this oncogene may contribute not only to early Wilms tumorigenesis, but may promote new vessel growth and resistance to normal apoptotic signaling during VEGF blockade. Thus, the activity of her2/neu in WT may serve as a paradigm for the interaction of tumor-specific oncogenes and VEGF-driven angiogenesis. These studies may improve the understanding of the contribution of her2/neu to WT angiogenesis, including its role in the response to VEGF blockade.

**Significance.** Recent clinical testing has validated VEGF as a therapeutic target in multiple human cancers\textsuperscript{14-16,23}. However, the apparent emergence of resistance during VEGF antagonism was not predicted by initial \textit{in vivo} experiments. This proposal attempts to address the general problem of acquired resistance to VEGF blockade, in the specific context of a common pediatric tumor. \textit{My role in these studies is to describe the response to changes in VEGF status in Wilms tumor as it relates to the level of her2/neu expression, with the ultimate goal of providing new therapies for children with aggressive disease.}

**APPLICANT'S SPECIFIC ROLE IN EXPERIMENTAL PLAN**

**Aim # 1:** We will determine whether her2/neu expression confers a relative survival advantage during VEGF blockade, by comparing the effects of blockade in xenografts expressing different levels of this receptor.

**We will relate her2/neu status to changes in vessel structure in WT.** In pilot studies, her2/neu expression was heterogeneous in xenografts derived from her2/neu(+) WT cells. We hypothesize that her2/neu expression may affect the ability of WT to survive hypoperfusion. It is possible that her2/neu(+) tumors will require less perfusion and fewer vessels to proliferate, analogous to the preferential survival of p53+/+ colon cancer cells in hypoxic tumor regions\textsuperscript{24}. Furthermore, since her2/neu independently regulates Ang-2\textsuperscript{20}, altered her2/neu status may affect endothelial attributes regulated by angiopoietins (sprouting, survival signaling). Therefore, we will use a validated \textit{general strategy} for examining tumor angiogenesis (summarized below) to compare xenografts derived from human WT cell lines which express high and very low levels of her2/neu (CU-UH1 and SK-NEP-1, respectively).

**Xenograft model:** We have previously described an orthotopic, metastasizing model of WT\textsuperscript{25}. Briefly, 10\textsuperscript{6} cultured human WT cells are injected intrarenally in athymic mice. Primary renal tumors develop in 97-100%, and grossly resemble clinical WT (e.g. lung metastasis). Her2/neu status of the xenografts will be verified by immunostaining with a her2/neu polyclonal antibody (DAKO, Carpinteria, CA) and by RT-PCR.

**General strategy for examining tumor vasculature:** we will quantify both overall vessel density in xenografts, as well as apoptosis/proliferation in vascular cells, by applying computerized image analysis. We will compare relative expression of angiogenic genes and assess survival signaling within tumor endothelium.

- **Vessel perfusion studies** – vessels are perfused with the fluorescein-labeled lectin \textit{Lycopersicum esculentum}, for quantitative assessment of vessel networks (density, branching, vessel ends, and total vessel length).
- **Angiogenic gene expression** – relative expression of VEGF, VEGFR2, VEGF-C, EphrinB2, EphB4, PIGF, Ang-1, Ang-2, Tie-2, PDGF-B in WT xenografts are determined by real-time RT-PCR, using normal mouse kidney as a control. Since localization of these genes may be relevant to cytokine function in tumors, expression is also assessed by \textit{in situ} hybridization.
- **Endothelial survival signaling** – to assess the status of Ras/MAPK and phophatidylinositol-3 kinase (P13-K) survival signaling pathways, PECAM immunostaining is combined with double-labeling for the phosphorylated forms of downstream MAPK and Akt, respectively, using commercially available antibodies.

**We will test the effect of blocking VEGF in xenografts expressing different levels of her2/neu.** The status of her2/neu may modulate the inhibitory effect of VEGF blockade, resulting in less effective growth or metastasis suppression in her2/neu(+) tumors. Using xenografts as above, we will use the validated decoy receptor construct VEGF-Trap (Regeneron Pharmaceuticals, Tarrytown, NY) for 5 weeks prior to sacrifice. Alteration in her2/neu expression will be studied by RT-PCR, and spatial relation of her2/neu(+) cells to
vasculature examined by combining her2/neu with EC immunostaining. We will employ the general strategy for examining tumor vasculature, outlined above, to determine how VEGF blockade alters tumor angiogenesis.

We will examine the effect of VEGF blockade on apoptotic signaling in xenografts expressing high and very low levels of her2/neu. Her2/neu disrupts proapoptotic signaling in a p53-independent manner in some breast cancer cells\(^{21}\). However, her2/neu also acts to decrease wild-type p53 function, via a PI3-K-dependent pathway\(^{22}\). Akt is phosphorylated, causing nuclear translocation of MDM-2 (Fig. 1). It is possible that this mechanism protects WT cells that overexpress her2/neu from apoptosis during environmental stress (i.e., hypoperfusion). To examine this possibility, we will compare Akt and MDM-2 in xenografts expressing high and very low levels of her2/neu, both at baseline and after VEGF blockade. Phosphorylated Akt will be detected and subcellular location of MDM-2 determined in tumor cells.

Aim #2: We will characterize the effects of her2/neu status on angiogenic factors in vitro and in vivo, and determine whether her2/neu inhibition affects response to VEGF antagonists.

We will determine if her2/neu regulates the expression of angiogenic factors in Wilms tumor in vitro. While her2/neu is known to upregulate VEGF and Ang-2 in various cancers\(^{19,20}\), its effect on other angiogenic factors (e.g. Ang-1, PDGF-B, EphrinB2) is not known. Our preliminary results suggest that these angiogenic factors may play a role in the vascular remodeling that supports tumor recurrence during chronic VEGF blockade. We will culture WT cell lines with different levels of her2/neu expression in the presence of both ligand (heregulin) and blocking antibody (Herceptin) to her2/neu. Expression of angiogenic factors will be determined by RT-PCR, using the breast cancer cell line MCF-7 (known to overexpress Her2/neu) as a positive control. The goal of these studies is to determine whether her2/neu signaling directly regulates angiogenic cytokines in WT cells.

We will examine the effect of her2/neu expression on Wilms tumor signaling in vitro. There is evidence for disruption of proapoptotic signaling by her2/neu via both p53-dependent and p53-independent mechanisms\(^{21,22}\). Blockade of the MEK/MAPK pathway can reverse taxol resistance in her2/neu-overexpressing breast cancer cells\(^{26}\). Yet her2/neu also acts to decrease wild-type p53 via the PI3-K pathway, which secondarily promotes VEGF synthesis\(^{22}\). It is not clear which of these mechanisms dominates the apoptotic signaling in WT. Therefore, we will inhibit the PI3-K pathway with LY294002 and wortmannin, and inhibit MEK signaling with PD98059 and U0176, just prior to the addition of ligand and blocking antibody to the described WT cell lines. We will measure apoptosis/proliferation and expression of angiogenic factors as described in the general strategy. These studies will provide new information regarding the roles of the MEK/MAPK and PI3-K pathways in her2/neu function in WT in vitro.

We will determine if her2/neu blockade alters tumor growth and angiogenesis in vivo, and how these effects compare to direct inhibition of VEGF. Recent evidence suggests that her2/neu acts to increase VEGF synthesis by increasing synthesis of HIF-1α, a mechanism distinct from that of p53 dysfunction (which acts to inhibit HIF-1α degradation)\(^9\). Thus, her2/neu blockade may cause decreased synthesis of HIF-1α, with resultant decrease in VEGF expression. The vascular phenotype resulting from constitutive overexpression of HIF-1α is strikingly different from that occurring after overexpression of VEGF\(^{27}\). Therefore, it is possible that blocking her2/neu will affect tumor angiogenesis differently than antagonism of VEGF. We will induce xenograft tumors with cell lines having different levels of her2/neu expression and administer vehicle or neutralizing anti-her2/neu antibody (humanized monoclonal anti-her2/neu antibody, Herceptin; Genentech) for
5 weeks. Using the general strategy, we will compare the effects of her2/neu blockade on vasculature (1) in each of these xenografts, and (2) to changes seen in each after VEGF blockade (Aim #1). The goal of these studies is to determine the effects of blocking her2/neu function on markers of tumor growth and specific attributes of vasculature, and to compare these to results of direct inhibition of VEGF.

**We will determine if her2/neu modifies the characteristics of tumors resistant to blockade of VEGF in vivo.** Preliminary studies showed that WT xenografts which became resistant to long term VEGF blockade displayed altered expression of angiogenic cytokines. Blockade of her2/neu downregulates VEGF\(^{11}\). Hence, it is possible that addition of anti-her2/neu blocking antibody may modify the response of WTs to VEGF antagonism. If VEGF or other angiogenic factors are downregulated by blockade of her2/neu, growth of vessels capable of supporting resistant tumor growth may be impaired. Therefore, we will superimpose her2/neu blockade on experiments designed to elicit resistance to VEGF antagonism. We will induce xenograft tumors in athymic mice, and after confirming that established tumors exist and are measurable at 5 weeks, will (1) sacrifice a cohort from each xenograft type as pre-intervention controls and (2) administer intraperitoneal vehicle, anti-her2/neu blocking antibody (Herceptin), VEGF-Trap, or a combination of the two agents. Animals will be monitored for tumor growth biweekly with calipers, and euthanized when the greatest tumor axis reaches 2.0 cm. Injections will continue until all animals are sacrificed or until no remaining animals have displayed tumor growth for 6 months (plateau). Tumors will be compared according to the general strategy. These studies aim to assess whether blockade of an oncogene contributing to VEGF upregulation will alter the characteristics of acquired resistance to anti-VEGF agents.

**RESEARCH ENVIRONMENT**

**Laboratory.** Dr. Jessica Kandel has 1000ft\(^2\) of laboratory space in the newly constructed Irving Cancer Research Building (Columbia University Medical Center). The space includes all equipment needed for this project, including tissue culture facilities, operating hood and microscope, equipment for immunohistochemistry, Applied Biosystems real-time PCR apparatus, and a fluorescent microscope with attached digital camera.

**Intellectual Resources.** I will attend the Introductory Angiogenesis lecture series offered by the Angiogenesis Group at Columbia (of which our lab is a member); weekly lab meetings (Thursday, 2-4 pm); 2 focused individual sessions with Dr. Kandel each month in addition to daily hands-on teaching in the lab; and Institute for Cancer Genetics seminars each Tuesday (12-1 pm).

**REFERENCES**


18. Dome DJ. Personal communication. Approximately 30.4% of archival specimens of Wilms tumor were positive by immunostaining for her2/neu. In.


RESEARCH ENVIRONMENT
My laboratory is one of three that form the Angiogenesis Group in the Institute for Cancer Genetics at Columbia University. The other two other investigators are Dr. Darrell Yamashiro (Pediatric Oncology and Pathology) and Dr. Jan Kitajewski (Pathology and Obstetrics and Gynecology). The clustered laboratories occupy 3000 adjacent square feet, of which 1000 is assigned to my lab, in the newly constructed Irving Cancer Research Building of Columbia University Medical Center. This building is a state-of-the-art research facility reserved for NCI-funded cancer biologists, with a dedicated vivarium. Institute for Cancer Genetics core facilities include a microarray center, FACS, and a confocal microscope. My individual space includes all equipment needed for this project, including tissue culture facilities, operating hood and microscope, equipment for immunohistochemistry, Applied Biosystems real-time PCR apparatus, and a fluorescent microscope with attached digital camera.

Columbia University Medical Center in general, and the Institute for Cancer Genetics in particular, provide an exceptionally rich environment for research. Included researchers compromise a broad cross-section of disciplines, ranging from computational biology to bioengineering to molecular genetics. More unusually, this group of investigators includes a mix of basic biologists and physician-scientists, and projects correspondingly range from basic inquiry to translational research. In addition, there are a wealth of seminars and endowed lectures daily. The library has a wide collection of journals, most of which are available online.
# Funding Information Report for

**Jason C Fisher**  
(JALI1931)  
Columbia University

## NIH Loan Repayment Programs

### Section 1 - Non-Profit or Government Research Funding Sources – Not From NIH

**Non-Profit Funding Source #1**

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### Section 2 – National Research Service Fellowship Award (NRSA) Support

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### Section 3 - NIH Grant Support

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### LRP PERSONAL STATEMENT

**NAME**

Jason C. Fisher, MD  

**DATE**

11/17/2007  

**PROJECT TITLE**

Combined Cyclooxygenase-2 and VEGF Blockade in Wilms Tumor

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I began surgical training with a firm work ethic and a dedication to children, and have nourished my clinical passions alongside an evolving research portfolio. Surgeons combine technical training with clinical medicine. This combination offers particular advantages, as awareness of clinical relevance is combined with the ability to perform technically challenging experiments. I will strive to become a true physician-scientist, and continue to build on the successes of my past 2 years of research on treatment-refractory pediatric cancer.

### Previous Research Training

When I drafted my initial LRP application two years ago, I was hopeful that my previous research training had prepared me for the challenges that lay ahead. Prior to my current fellowship, I had gained valuable experience from diverse areas of scientific study, including basic and clinical science, outcomes research, and computational modeling. Early in medical school, I joined the laboratory of Dr. Patricia Donahoe in the Department of Pediatric Surgery at the Massachusetts General Hospital. Funded by an NIH-HL grant, I used a rodent model of congenital diaphragmatic hernia (CDH) to show that fetal lung growth correlates with intracellular redox state. These results received top prize at the Association for Academic Surgery 2003 National Medical Student Research Competition. To address deficiencies in familial colorectal cancer (CRC) risk assessment among primary care providers, I created software for PDA devices to assist housestaff in determining CRC risk. Funded by an NCI grant, this software was used in a randomized clinical trial where it significantly improved the knowledge of familial CRC among clinicians. I began surgical residency in July 2003 at Columbia University, where the surgical faculty have nurtured my clinical judgment and technical skills as I train to become a highly-skilled academic surgeon. I continued expanding my research base through a project with Dr. Larry Bodenstein in the Department of Pediatric Surgery, and received two Startup Grant Awards from Columbia to develop a novel, cell-based computer model capable of simulating diaphragm embryology. We published simulations that revealed mechanistic flaws in the prevailing theories on diaphragm growth. Keeping continued focus on the care of children, I also assessed outcomes in pediatric thyroid cancer, and presented data on risk factors associated with recurrent thyroid cancer at the 2005 NY Surgical Society Scientific Session.

Having familiarized myself with broad avenues of research, I had outlined three short-term objectives in my first LRP application: (1) acquisition of new skills in molecular tumor biology, (2) learning how to critically evaluate experimental data, and (3) developing hypothesis-driven approaches to overcoming treatment-refractory Wilms tumor. Under direction of Drs. Jessica Kandel and Darrell Yamashiro, the Pediatric Tumor Biology Laboratory has been instrumental in helping me achieve these goals. My primary project centers on the hypothesis that combined VEGF and COX-2 blockade might perturb tumor vasculature more effectively than either approach alone (see Research Accomplishments). Using *in vivo* xenograft tumor models, I learned to apply my skills as a surgical resident to the microsurgical environment of animal protocols. I translated my past experience in computational modeling to new skills in 3D reconstruction of tumor vasculature and digital analysis of immunofluorescent staining. I gained understanding of how to critically evaluate research data as I prepared for institutional and national presentations, resulting in a 2007 Columbia University Blakemore Award for Research, additional Startup Grants, and a Poster of Distinction award at the 2007 meeting of the American Pediatric Surgical Association. I have benefited from working in a structured laboratory environment rich in mentorship and bolstered by weekly lab meetings, focused sessions with Dr. Kandel, and a diverse seminar series. Most importantly, this supportive environment has taught me to develop hypothesis-driven questions essential to my growth as a scientist and physician. My Renewal LRP proposal embodies this last skill set, as I have incorporated my research accomplishments of the last 18 months into focused Specific Aims (Research Activities section) that I can confidently pursue over the next 2 years.
Short-Term Research Objectives

Regarding my short-term research objectives, I plan to:

1. Investigate my new hypotheses on the role of VEGF and COX-2 in driving Wilms tumor metastasis using the skills obtained in the first two years of my research fellowship.
2. Develop additional expertise in advanced techniques of bioluminescent imaging, 3D reconstruction of vascular networks, and genetic microarray interpretation.
3. Adopt a senior role in the laboratory by mentoring new researchers, drafting comprehensive manuscripts, and designing new experiments to combat treatment-refractory pediatric tumors.

Over the next two years, I will complete general surgery training while continuing to participate in the laboratory. With increased clinical obligations, my ability to achieve these new short-term goals is dependent on a rigorously structured research framework. To maintain this clear distinction between clinical activity and my LRP weekly time commitment of 20 research hours, I have designed a research-time allocation outline with Dr. Kandel (Research Activities section) and will document my lab participation in an audit-ready online log. The digital imaging techniques and microarray analyses described above can readily be performed at hours that accommodate my schedule. As I mature into a senior investigator, I am eager to mentor new researchers and focus my own research activities on data interpretation and manuscript generation. I have taken early steps toward mentoring students, and was nominated as the Most Influential Investigator by a laboratory student participating in the 2008 Intel Science Talent Competition.

Long-Term Career Goals

My long-term career goals involve:

1. Becoming a capable research scientist, contributing to the study of treatment-refractory childhood diseases.
2. Augmenting my clinical and technical skills by seeking advanced training in pediatric surgery.
3. Emerging from training as a competent and motivated physician-scientist eligible for further research funding, and ultimately transitioning into a fully independent R01-funded investigator.

These long-term ambitions mirror those I set when I first began my current research fellowship. Overall, this experience has exponentially advanced my growth as a research scientist. My integration into the Department of Pediatric Surgery has afforded me the opportunity to publish various clinical papers as well. The structure of my next 2 years of training will provide further insight into the dichotomy of a surgeon-scientist career, as I will carefully balance both clinical obligations and a focused research agenda. Despite the challenges inherent to this career path, I am hopeful that my past and recent accomplishments will fuel future successes. I will complete general surgery residency in July 2010, with plans to apply for advanced training in pediatric surgery. Of Dr. Kandel’s previously-mentored 11 postdoctoral surgical research fellows, all have gone on to advanced academic training, including 7 training specifically in pediatric surgery.

My parents are public school teachers for New York City and have always struggled financially. I worked diligently to merit a full-tuition undergraduate scholarship to Boston University, but I incurred great debt in financing my medical education. I remain firm in my career goal of becoming a surgeon-scientist, and am extraordinarily thankful to the NIH for awarding me an LRP grant in 2006. Renewal of this support will be invaluable as I continue working with passion and diligence to battle childhood disease.
NAME
Jason C. Fisher, M.D.

POSITION TITLE
General Surgery Resident, PGY-3
Pediatric Surgery Postdoctoral Research Fellow, Year-2

EDUCATION/TRAINING

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<td>B.A.</td>
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<td>881 Commonwealth Avenue, Boston, MA 02215</td>
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<td>Boston University School of Medicine</td>
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A. Positions

06/2003 – 06/2006: General Surgery Resident, NY Presbyterian Hospital – Columbia University Medical Center
Milstein Hospital Building, 7-Garden-South, Rm 313
177 Fort Washington Ave, New York, NY 10032

07/2006 – 06/2008: Research Fellow, Division of Pediatric Surgery – Columbia University Medical Center
Morgan Stanley Children’s Hospital of NY Presbyterian, Babies-North, Rm 201
3959 Broadway, New York, NY 10032

Professional Certifications

2006: State Medical License – Renewal, New York State Office of the Professions
2006: Advanced Trauma Life Support – Recertification, American College of Surgeons
2006: State Medical License, New Jersey Office of the Attorney General
2007: Basic and Advanced Cardiac Life Support – Recertification, American Heart Association

Professional Memberships

1999: Current Member, American Medical Association
1999: Current Member, Massachusetts Medical Society
1999: Current Member, American Medical Students’ Association
2002: Current Member, Alpha Omega Alpha
2003: Current Member, New York State Medical Society
2003: Resident Member, Association for Academic Surgery
2004: Resident Member, American College of Surgeons

Honors and Grants

1996: Trustee Scholarship – Merit scholarship for full undergraduate tuition, Boston University
2001: Dean’s Accolade for Exceptional 2nd Year Performance, Boston University School of Medicine
2002: Honor Society Inductee, National Association of Pathology Chairs
2002: Honor Society Inductee, Alpha Omega Alpha
2003: Mary Stafford Award for Excellence in Surgery, Boston University Department of Surgery
2003: Top Prize, National Medical Student Research Award, Association for Academic Surgery
2003: Startup Grant Research Award, Columbia University Department of Surgery
2004: Startup Grant Research Award, Columbia University Department of Surgery
2006: Startup Grant Research Award, Columbia University Department of Surgery
2006: NIH-LRP Extramural Pediatric Research Grant, National Institutes of Health
2007: ABSITE Award for Highest In-Service Examination Score, Columbia University Department of Surgery
2007: Distinguished Scientific Poster Award, 38th Annual APSA Meeting, American Pediatric Surgical Assoc.
2007: Startup Grant Research Award, Columbia University Department of Surgery
2007: Blakmore Prize for Outstanding Surgical Research, Columbia University Department of Surgery

B. Peer-Reviewed Publications


**Invited Talks / Lectures**


**National Meeting Oral Presentations and Posters**


C. Research Support and Previous Experience

| Principal Investigator: | Jessica J. Kandel, MD – Department of Pediatric Surgery, Columbia University
| Project Status / Role: | Currently Participating – Resident Research Fellow, 2006-Present
| Research Goals: | To determine the impact of VEGF and COX-2 on angiogenesis in Wilms tumor
| Project Support: | Pediatric Cancer Research Foundation – Annual Grant, 2008
NIH R01 Grant – R01-CA124644 (Yamashiro)
NIH R01 Grant – R01-CA100451 (Kandel)
Surgical Research Startup Grant – Department of Surgery, Columbia University

| Principal Investigator: | Darrell J. Yamashiro, MD, PhD – Department of Pediatric Oncology, Columbia University
Project Status / Role: | Currently Participating – Resident Research Fellow, 2006-Present
Research Goals: | To determine the impact of VEGF and COX-2 on angiogenesis in Wilms tumor
Project Support: | Pediatric Cancer Research Foundation – Annual Grant, 2008
NIH R01 Grant – R01-CA124644 (Yamashiro)
NIH R01 Grant – R01-CA100451 (Kandel)
Surgical Research Startup Grant – Department of Surgery, Columbia University

| Principal Investigator: | Lawrence Bodenstein, MD, PhD – Department of Pediatric Surgery, Columbia University
Project Status / Role: | Completed Project – Resident Investigator, 2003-2006
Research Goals: | Modeling abnormal morphogenesis of the fetal diaphragm with novel computer simulation
Project Support: | Surgical Research Startup Grant – Department of Surgery, Columbia University

| Principal Investigator: | Kathryn Spanknebel, MD – Department of Surgery, Columbia University
Project Status / Role: | Completed Project – Resident Investigator, 2005-2006
Research Goals: | Compiled and analyzed outcome data for pediatric thyroid disease
Project Support: | New York Thyroid Center, Department of Surgery, Columbia University

| Principal Investigator: | Jay J. Schnitzer, MD, PhD – Department of Pediatric Surgery, Mass General Hospital
Project Status / Role: | Completed Project – Medical Student Research Associate, 2000-2003
Research Goals: | Identified redox as a key regulator of fetal lung growth in congenital diaphragmatic hernia
Project Support: | NIH HL Grant – HL-03132

| Principal Investigator: | James M. Becker, MD – Department of Surgery, Boston University School of Medicine
Project Status / Role: | Completed Project – Medical Student Research Associate, 2002-2003
Research Goals: | Examined the role of oxidative stress within a mucosa tissue model of ulcerative colitis
Project Support: | Department of Surgery, Boston University School of Medicine

| Principal Investigator: | Paul C. Schroy, MD, MPH – Department of Gastroenterology, Boston University
Project Status / Role: | Completed Project – Medical Student Research Associate, 2002-2003
Research Goals: | Designed software for PDA devices to assist in assessing colorectal cancer risk
Project Support: | NCI Grant – K22-CA90680
I have completed 18 months of a two-year research fellowship in the Pediatric Tumor Biology Laboratory directed by Drs. Jessica Kandel (Pediatric Surgery) and Darrell Yamashiro (Pediatric Oncology) in the Institute for Cancer Genetics at Columbia University. To combat treatment-refractory pediatric cancers, my primary project has focused on how Wilms tumor develops resistance to sustained vascular endothelial growth factor (VEGF) blockade, and has been supported by two NIH R01 grants held by Drs. Kandel and Yamashiro.

While inhibiting VEGF suppresses tumor growth by disrupting endothelial cells, tumors exposed to prolonged VEGF blockade ultimately recur and display enhanced recruitment of perivascular support cells. We previously showed that vascular mural cell (VMC) recruitment is attenuated by inhibiting the cyclooxygenase-2 (COX-2) pathway. I hypothesized that combined VEGF/COX-2 blockade might perturb tumor vasculature more effectively than either approach alone. I addressed this by inducing intrarenal xenografts by injection of $10^6$ cultured human Wilms tumor cells (SK-NEP-1) in athymic mice. Animals received one of 4 treatments: (1) vehicle control; (2) selective COX-2 inhibitor (SC-236); (3) anti-VEGF antibody (bevacizumab); or (4) combined SC-236 + bevacizumab. These studies showed suppressed tumor growth in all treatment groups, with comparable growth inhibition noted in the bevacizumab and combination therapy groups. Immunohistochemistry and 3D imaging revealed that tumors treated with combined agents displayed hypetruncated vessels, defective branching hierarchy, and reduced VMC recruitment (Fig. 1). These results suggest that dual endothelial/VMC targeting may more effectively disrupt tumor angiogenesis, potentially providing more sustained therapeutic responses. I presented these data at the 38th meeting of the American Pediatric Surgical Association, and received the conference Poster of Distinction award. Additionally, the Columbia University Department of Surgery awarded me a 2007 Blakemore Award for Outstanding Surgical Research, along with a Startup Grant to further investigate these findings.

Further analysis of these data revealed synergy between SC-236 and bevacizumab in reducing lung metastases. While existing data support individual roles of COX-2 and VEGF as mediators of tumor metastasis, mechanisms linking combined COX-2/VEGF status to metastatic potential in pediatric tumors remain unclear. In the next 6 months, I will perform microarray analysis on treated tumors to uncover gene expression differences relevant to metastatic potential. These efforts coincide with the next phase of my project (Research Activities section), which involves two specific aims: (1) Determining how COX-2/VEGF inhibition affects individual steps of the metastatic process; and (2) differentiating between the tumor and host-mediated mechanisms contributing to metastasis suppression during combined COX-2/VEGF blockade.

![Figure 1: Vessel analysis from Wilms tumor xenografts treated with antagonists of COX-2 (SC236) and VEGF (bevacizumab), 3D reconstructions are based on confocal image data. Immunohistochemistry for α-smooth muscle actin (αSMA) revealed dilated, erratic vessels with decreased VMC in SC236-treated tumors; conversely, bevacizumab-treated tumors displayed increased VMC. Combination-treated tumors showed highly perturbed architecture with decreased VMC and defective branching.](image-url)
I have devoted attention to additional projects which similarly focus on overcoming resistance to sustained angiogenic inhibition. Our laboratory has shown that orthotopic pediatric tumor models can uniquely reflect tumor-specific patterns of growth and metastasis that are useful for validating new therapies. However, real-time in vivo information on xenograft size, location, viability, and metastasis is difficult to acquire, limiting longitudinal assessment of treatment responses. To optimize our ability to obtain such real-time data, I have helped stably-transfect Wilms tumor and neuroblastoma cell lines with the firefly luciferase gene, and have used these cells to pilot our first experience with two and three-dimensional bioluminescent imaging. We have integrated this technique into a novel multi-modality imaging protocol that also includes ultrasonography and magnetic resonance imaging. My preliminary results (Fig. 2) suggest that combining modalities can impart useful data about metastasis, local invasion, and viability, allowing real-time assessment of longitudinal responses to novel treatments in solid tumor models. We have submitted these results for presentation at the 39th meeting of the American Pediatric Surgical Association.

My accomplishments over the last 18 months have generated useful data on the effects of combined anti-angiogenic therapy on tumor vasculature and metastasis in a common pediatric tumor. I have also helped design a multi-modality imaging protocol to optimize data generated by orthotopic tumor models. With abstracts accepted for presentation and manuscripts currently in preparation, these efforts have received awards at the institutional and national level, and have led to new hypotheses regarding the mechanisms driving tumor metastasis. The specific aims outlined in the Research Activities section will build on these successes, and may provide a rationale for use of combination anti-angiogenic therapies in treatment-refractory pediatric tumors.
SUMMARY

While most children with Wilms tumor (WT) are cured, a subset will develop life-threatening pulmonary metastases. Development of hematogenous metastasis involves a complex sequence of tumor-host interactions which depend on new blood vessel growth (angiogenesis) at both primary tumor and metastatic sites. Recently, angiogenesis inhibitors have been clinically validated as treatment for metastatic disease in various human cancers, and may offer promise for children with refractory WT. Blockade of the pro-angiogenic vascular endothelial growth factor (VEGF) disrupts endothelial cell recruitment and decreases rates of distant metastasis in experimental tumor models. We have previously shown that WT ultimately becomes resistant to VEGF blockade, suggesting that an alternative mechanism may support angiogenesis and metastasis progression during sustained VEGF inhibition. Blockade of the inducible enzyme cyclooxygenase-2 (COX-2) is known to suppress the growth of multiple human cancers. We recently demonstrated that COX-2 blockade impairs WT growth in part by attenuating the incorporation of vascular mural support cells into tumor vessels. Recent data has also linked COX-2 expression to metastatic progression in certain human tumors. Collectively, these findings suggest that combined VEGF and COX-2 blockade may more effectively prevent metastasis than either approach alone. We tested this hypothesis under the support of my initial NIH Pediatric Research LRP (see Research Accomplishments section). These initial experiments revealed a synergistic effect between VEGF and COX-2 inhibitors on the rate of lung metastasis in a WT xenograft model. In this Renewal LRP proposal, we will study the potential mechanisms involved in VEGF and COX-2 mediated tumor metastasis. We will determine which specific steps in the metastatic process are affected by changes in COX-2 and VEGF status. We will characterize the differential responses of tumor and host compartments to combined VEGF and COX-2 blockade with respect to lung metastasis in a WT xenograft model. Our overall goal is to develop effective new treatments for children with WT by identifying and then overcoming the mechanisms by which these tumors develop lethal metastases.

BACKGROUND AND SIGNIFICANCE

**Wilms tumor (WT) is the second most common primary abdominal tumor in children.** While most children are cured with current regimens, mortality remains high among the 10% who develop hematogenous metastasis. These patients urgently require new therapies, and might benefit from novel strategies that target pathways involved in metastatic progression. No single genetic marker defining the metastatic potential of WT has been identified. However, certain molecular alterations are detected in high-risk WT (e.g. loss of heterozygosity), and a lung metastasis gene signature (LMS) has recently been identified in adult cancers as predictive of treatment-refractory metastatic disease. Additionally, innate host factors may influence tumor metastasis, possibly through bone marrow-mediated creation of a pre-metastatic niche at distant metastatic sites. Thus, multiple factors are likely to be involved in the development of hematogenous metastasis in WT. We hypothesize that the capacity to develop vasculature with particular features that support the metastatic process may be an underlying common theme in aggressive disease.

**Acquiring the capacity to induce a new blood supply is a crucial step in tumor metastasis.** Tumor cells intravasation, survival in circulation, extravasation into a distant organ, angiogenesis, and uninhibited growth are essential requirements for metastasis. New blood vessel growth depends on the expression of pro-angiogenic cytokines. VEGF, the best understood, promotes endothelial cell (EC) proliferation and survival primarily through VEGF-receptor-2 (VEGFR2) activation, and is nearly ubiquitous in human tumors. Elevated VEGF expression is associated with metastasis and poor clinical prognosis in WT, and a subset of LMS genes are known to be downstream effectors of VEGF (e.g. MMP-1, MMP-2, COX-2). Additionally, VEGFR1-positive bone marrow progenitors have been shown to precede the arrival of tumor cells at pre-metastatic sites.
**VEGFR blockade has been clinically validated as an antiangiogenic strategy in metastatic disease**\(^1\-3,28\). Our previous \textit{in vivo} studies have shown that tumor growth and lung metastases are decreased in VEGF-blocked WT\(^5\,8\,27\). However, prolonged VEGF antagonism ultimately leads to recurrent tumor growth in WT xenografts\(^9\). Tumors escaping VEGF blockade demonstrate perturbed vascular architecture characterized by enhanced recruitment of perivascular support cells, suggesting a role for alternative pathways that support angiogenesis and metastasis progression. Dissecting these complementary angiogenic mechanisms may provide novel data about processes governing vascular integrity and metastasis in WT during VEGF blockade.

**Cyclooxygenase-2 (COX-2), an inducible enzyme that catalyzes prostaglandin synthesis, promotes multiple aspects of tumor angiogenesis**\(^28,29\). COX-2 has multiple functions in tumor pathogenesis, and overexpression correlates with poor clinical prognosis in many human cancers\(^30,31\). Selective COX-2 inhibitors have markedly suppressed tumor growth and angiogenesis in preclinical studies\(^32,33\), but conflicting data exist regarding the pro-angiogenic mechanisms of COX-2. Initial evidence suggested that COX-2 exerts a VEGF-dependent effect on endothelial cells\(^32,34\). However, we recently demonstrated that COX-2 can influence the recruitment of vascular mural cells (VMC) into developing tumor vasculature through mechanisms independent of VEGF (e.g. chemokine receptor CXCR4 and platelet-derived growth factor (PDGF-B) signaling)\(^12\). We hypothesized that addition of COX-2 inhibition to VEGF blockade would synergistically target the VMC and endothelial compartments and more effectively suppress tumor growth than either approach alone.

**Combined COX-2 and VEGF blockade markedly suppresses pulmonary metastasis.** In pilot studies, we found that combined COX-2 and VEGF blockade, while uniquely perturbing the vessel architecture of WT xenografts, suppressed overall tumor growth to the same extent as achieved with anti-VEGF monotherapy. However, we observed a significantly lower incidence of lung metastasis in xenografts treated with both COX-2 and VEGF antagonists compared to tumors treated with either agent alone (\(P<0.05\), Kruskal-Wallis analysis). These findings are consistent with the recent identification of COX-2 as part of the LMS gene family\(^13,19\), but it remains unclear as to which phase of the metastatic process is most profoundly influenced by combined inhibition. These studies raise the possibility that COX-2 acts to reduce metastasis by altering tumor cell invasiveness, modifying permeability of tumor and lung vasculature, or perturbing CXCR4 and CXCR7 chemokine signaling (which is known to home tumor cells to the lung). Our findings suggest that both tumor and host elements contribute to metastatic progression in WT, and that combined COX-2 and VEGF blockade may exert differential effects on each compartment. These studies may improve the understanding of how metastatic pathways can be manipulated to maximize the effect of combination therapies.

**Significance.** Recent clinical testing has validated VEGF as a therapeutic target in metastatic cancers\(^1\-3,26\). COX-2 may regulate elements of angiogenesis and tumor behavior which promote tumor metastasis, making it an attractive therapeutic adjunct to VEGF blockade. In this proposal, we will attempt to address the general problem of metastasis as it relates to distinct pathways that regulate vascular proliferation and function, in the specific context of a common pediatric tumor. My role in these studies is to describe how different components of the metastatic process respond to changes in COX-2 and VEGF status in Wilms tumor, with the ultimate goal of providing new therapies for children with aggressive disease.

**SPECIFIC AIMS**

**Aim #1:** We will determine how COX2 and VEGF inhibition influence the discrete steps (intravasation, extravasation, growth at distant site) which constitute the metastatic process in WT.

\textit{We will determine whether WT cell migration through endothelium is influenced by COX-2 status in vitro.} Establishment of hematogenous metastases is dependent on the ability of tumor cells to invade their neovasculature (intravasation) as well as migrate out of pulmonary capillary beds (extravasation). Reduction in metastases observed during COX-2 inhibition\(^13\-15,19\) may reflect an altered ability of tumor cells to penetrate a vascular endothelial barrier. We will use a transendothelial migration assay to assess the ability of cultured human WT cells (SKNEP-1) to traverse an endothelial monolayer (cultured HUVECs) in the presence of graded concentrations of the celecoxib analog SC-236.
We will test whether extravasation of WT cells into lung tissue is affected by changes in COX-2 and VEGF status in vivo. Factors influencing extravasation of circulating tumor cells at distant sites may be independent of the processes which regulate intravasation at the primary tumor. To examine how COX-2 and VEGF status specifically affect extravasation of tumor cells into the lung, we will pretreat athymic mice with Human-Fc protein, SC-236 + Human-Fc, bevacizumab (humanized monoclonal anti-VEGF antibody), or combined agents. After one week of pretreatment, we will inject SKNEP-1 cells engineered to express luciferase via the tail vein. Metastasis will be assessed in vivo using bioluminescent imaging with a charge-coupled device camera, and animals euthanized when metastatic burden reaches $3 \times 10^9$ photons/sec.

We will relate changes in the incidence and size of pulmonary metastases to permeability of lung vasculature. Metastatic growth involves both the arrival of tumor cells in the lung (extravasation, survival in lung parenchyma) which is reflected as the incidence of metastatic deposits, and effective progressive growth of these lesions (which requires effective delivery of oxygen and nutrients, either via diffusion or vasculature). To relate the transit of macromolecules during metastasis, we will inject both Fc protein and biotinylated albumin into the 4 cohorts of pretreated athymic mice (as above) and examine (1) presence of Fc protein in lung tissue and (2) proportion of labeled albumin extravasated to lung.

We will examine differences in chemokine and angiogenic gene expression in relation to pulmonary vasculature just prior to the arrival of metastatic tumor cells. We will euthanize a subset of mice from each pretreatment cohort of the tail-vein injection experiment and sacrifice prior to tumor cell injection, to determine how COX-2 and VEGF inhibition affect gene expression in relation to the pulmonary vasculature prior to tumor cell arrival. Next, we will assess the ability of COX-2 and VEGF to alter the premetastatic niche by pretreating animals as above, with the addition seven daily injections of serum-free conditioned media from cultured SKNEP-1 cells. Matched controls for each pre-treatment cohort will receive unmodified serum-free media. We will use a validated General Strategy for examining angiogenesis (summarized below) to compare the lung parenchyma in mice pretreated with COX-2 and/or VEGF antagonists.

General Strategy for examining vasculature: we will quantify overall vessel density and specific vascular attributes in lung tissue by applying computerized image analysis. Additionally, we will use microarray and quantitative PCR to compare relative expression of angiogenic and metastasis-relevant genes.

- **Vessel perfusion studies** – vessels are perfused with the fluorescein-labeled lectin *Lycopersican esculentum*, for quantitative assessment of vessel networks (density, branching, vessel ends and total length).
- **Immunohistochemistry** – specific vascular attributes will be analyzed based on endothelial and vascular smooth muscle antigens. We will use phosphospecific antibodies to determine the status of pVEGFR2 and pPDGFRβ in vessels, as well as patterns of VEGF and PDGF expression. We will assess the distribution and status of CXCR4/SDF-1 and CXCR7/CXCL11 signaling axes, as well as known downstream targets of COX-2.
- **Microarray analysis** – RNA will be isolated from lung tissue and prepared for Affymetrix Genechip analysis to identify differentially expressed genes in each cohort. Relative expression of angiogenic and metastasis-relevant genes will be quantitatively-determined by RT-PCR, using normal mouse lung or kidney as a control.

Aim # 2: We will differentiate between the tumor versus host-mediated mechanisms that contribute to the suppression of WT metastasis observed during combined COX-2 and VEGF inhibition.

We will determine whether synergistic metastasis suppression occurs during combined inhibition if COX-2 antagonism is limited only to the tumor compartment. We will establish a stable knockdown of COX-2 in SKNEP-1 cells through shRNA transfection. Next, we will induce four cohorts of intrarenal xenografts: (1) control, (2) COX-2 knockdown, (3) VEGF blockade, and (4) COX-2 knockdown plus VEGF blockade. Xenografts in control and VEGF monotherapy groups will be established using SKNEP-1 cells transfected with an empty vector. This design mirrors our previous 4-cohort study looking at combined inhibition in a xenograft model (outlined in Research Accomplishments). However, COX-2 inhibition in this initial study was achieved with an oral SC-236, which affected both the tumor and host compartments. By using COX-2 shRNA, we can determine whether the metastasis suppression observed in our initial study is primarily the result of manipulations to the tumor versus the host compartment.
We will determine whether COX-2 mediated effects on transendothelial migration reflect changes in tumor cell invasiveness versus alterations to the endothelial barrier. We will perform the transendothelial migration assay described in Aim #1 to determine whether SKNEP-1 cells transfected with COX-2 shRNA can traverse an endothelial monolayer as effectively as SKNEP-1 cells transfected with an empty vector. This will distinguish whether COX-2 mediated alterations of tumor cell intravasation result from changes in tumor cells versus the endothelium.

We will examine the effect of VEGF blockade on extravasation of COX-2 deficient WT cells into lung tissue. As described in Aim #1, we will pretreat athymic mice with Human-Fc protein or anti-VEGF antibody for one week, and then inject the tail vein with luciferase-expressing SKNEP-1 cells transfected with either COX-2 shRNA or an empty vector. Metastasis will be assessed in vivo using bioluminescent imaging, and animals euthanized when metastatic burden reaches 3 x 10^6 photons/sec.

Applicant’s Specific Role in Aims #1 and #2:
I anticipate increased clinical responsibilities starting July 1, 2008, as I continue my general surgery training in pursuit of a career as a highly-skilled pediatric surgeon. To ensure I devote 20 hours each week exclusively to research activity, I have outlined with Dr. Kandel specific project applications where I can generate data on an independent schedule, thereby optimizing my research hours in a more flexible time format. Accordingly, my role in these Specific Aims will chiefly consist of digital image analysis (flux mapping of bioluminescent images, immunohistochemistry, 3D reconstruction of confocal imaging), microarray data interpretation, and manuscript preparation. These tasks are readily performed outside standard laboratory hours with minimal clinical interference. While exact research time allocations will vary each month based on project status and available data, Table 1 outlines my general participation strategy. To provide an audit-ready record of my participation for both Dr. Kandel and the NIH, I will maintain a time-stamped electronic research log at http://cumc.columbia.edu/dept/surgery/peds/LRP.html, which will track my research hours and activities in a similar fashion as continuing medical education (CME) credits are logged for the Department of Surgery faculty. By establishing a structured research framework, we will better maintain a clear distinction between my clinical duties and my NIH-LRP weekly research time commitment.

RESEARCH ENVIRONMENT

Laboratory and Intellectual Resources. Dr. Kandel has 1000ft² of laboratory space in the newly constructed Irving Cancer Research Building (Columbia University Medical Center). The space houses all equipment needed for this project, including tissue culture and mouse barrier facilities, immunohistochemistry supplies, real-time PCR apparatus, and a fluorescence microscope with digital camera. A bioluminescent imaging camera is available in the mouse barrier facility. All digital analysis software and microarray data is remotely accessible through remote server connections, facilitating active participation in research activities from all over the Columbia University campus. I will attend weekly laboratory meetings (Tuesday, 2-4 pm). Focused individual sessions with Dr. Kandel will take place each week to ensure project goals are being met and that compliance with research hours is being adequately documented in the online log.

REFERENCES
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