Creighton University Cancer and Smoking Disease Research Program LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

INTRODUCTION AND SUMMARY
Thomas F. Murray, PhD, Principal Investigator

Creighton University is pleased to submit this annual report to the State of Nebraska regarding the activities and advancement of its Cancer and Smoking Disease Research Program, funded by the State of Nebraska Cancer and Smoking Disease Research Program (LB595). This progress report provides details on the Administration and Planning Program, Development Program, and the continuing major research programs (Molecular Mechanisms and Novel Targets in Cancer, Discovery of Novel Mutations in Hereditary Cancer, and Molecular and Cellular Mechanisms of Smoking-Related Lung Disease).

As documented in the program reports, the Cancer and Smoking Disease Research Program Year 21 has been productive for the investigators at Creighton University. During the reporting period, the Discovery of Novel Mutations in Hereditary Cancer Program published 23 refereed publications, the Molecular Mechanisms and Targets in Cancer Program produced 5 refereed publications, and the Molecular and Cellular Mechanisms of Smoking-Related Lung Disease Program produced 4 refereed publications. These manuscripts were published in such high-caliber journals as *Nature Reviews Cancer*, *European Journal of Cancer*, *Cancer Discovery, Breast Cancer Research*, and *American Journal of Respiratory Cell and Molecular Biology.*

Creighton University's Cancer and Smoking Disease Research Program has been extremely effective at leveraging the State of Nebraska's support into extramural funding over the past 21 years. The program has served as means to develop and expand important research projects. This support has provided Creighton the resources to develop investigators who then seek funding from other sources, such as the National Institutes of Health. During this period, the State has contributed $28,910,340 to Creighton University through LB595. This, coupled with Creighton's contribution of $12,865,101 through unrecovered indirect costs and $23,593,067 in Health Future Foundation funding, has led to $144,460,542 of extramural funding brought into Creighton University and the State of Nebraska. The return on the State of Nebraska's investment has therefore been exemplary, with each dollar of LB595 leading to $5 in extramural funding for Creighton University. This five-fold return on the investment clearly demonstrates the effectiveness of Creighton faculty in leveraging the LB595 support.

Meeting and member details for the Executive, Internal Advisory, and External Advisory Committees are included in the Administration and Planning Program Progress Report. The Publications included in the program reports represent all those germane to the respective programs.

Total awards received by LB595 participants from inception of program (July 1, 1994 - June 30, 2015)

Participants	External Awards	Other Internal Awards			LB 595	Unrecovered Indirects on LB595	Total
		HFF	LB692	Haddix President's Award			
Adrian, Thomas	1,516,191				1,892,953	842,364	4,251,508
Abel, Peter	446,261		100,000		119,239	53,061	718,561
Arouni, Amy	333,610	19,385	75,000		119,999	53,400	601,394
Bagchi, Debasis	326,833	10,000			18,580	8,268	363,681
Bagchi, Manashi	5,000				175,942	78,294	259,236
Bergren, Dale	94,917				93,336	41,535	229,788
Bockman, Charles	120,944	30,000			80,000	35,600	266,544
Brauer, Philip	1,035,556	-			79,088	35,194	1,149,838
Brumback, Roger		410,758	534,363		330,500	147,073	1,422,694
Casale, Thomas	11,866,522	1,897,347	90,000		420,000	186,900	14,460,769
Chakkalakal, Dennis	43,600	9,921	33,251		80,000	35,600	202,372
Chen, Xian-Ming	2,927,579	390,827	547,132		480,000	213,600	4,559,138
Cullen, Diane	3,459,396	351,552	75,000		1,450,873	645,638	5,982,459
Dash, Alekha	376,603		99,036	10,000	15,591	6,938	508,168
Deng, Hong-Wen	2,507,316	35,069	923,693		438,806	195,269	4,100,153
Dewan, Naresh	184,639				20,000	8,900	213,539
Dey, Bhakta	509,025	20,000	285,000		40,000	17,800	871,825
Dravid, Shashank	1,388,552	221,206	75,000	30,000	120,000	53,400	1,888,158
Drescher, Kristen	4,260,498	316,000	1,805,367		666,985	296,808	7,345,658
Edwards, John	43,294	316,647			19,953	8,879	388,773
Enarson, Cam	12,637,502	9,062,817	405,075		863,292	384,165	23,352,851
Filipi, Charles	1,044,750	81,634			19,625	8,733	1,154,742
Foster, Jason		233,579			335,000	149,075	717,654
Gatalica, Zoran					61,147	27,210	88,357
Gentry-Nielsen, Martha	721,421	5,100			80,000	35,600	842,121
Govindarajan, Venkatesh	1,887,395	40,000	319,798	15,000	547,622	243,692	3,053,507
Hagenkord, Jill		20,000	100,000		75,000	33,375	228,375
Hansen, Laura	3,352,949	79,897	877,300		1,090,000	485,050	5,885,196
Harrison, Christopher	738,723	16,485			61,977	27,580	844,765
Haynatzki, Gleb	85,741				107,135	47,675	240,551
Heaney, Robert	9,202,964	1,343,251	50,212		185,112	82,375	10,863,914
Hinder, Ronald					19,859	8,837	28,696
Hodgson, Clague	543,300				522,902	232,691	1,298,893
Hogenmiller, Jette					7,117	3,167	10,284
Johnson, Mark		15,000			30,000	13,350	58,350
Khan, Manzoor	352,400				39,970	17,787	410,157
Knezetic, Joseph	76,000	395,100			761,420	338,832	1,571,352
Lefkowitz, David	108,271				20,000	8,900	137,171
Loggie, Brian			40,000		300,000	133,500	473,500
Lovas, Sandor	1,591,866	309,822	6,257		293,961	130,813	2,332,719
Lynch, Henry	17,938,532		100,000		4,770,997	2,123,094	24,932,623
Mackin, Robert	1,433,955	42,800			235,898	104,975	1,817,628
Mailliard, James	994,796				20,000	8,900	1,023,696
Mansky, Louis	92,176	10,000			108,182	48,141	258,499
Mohiuddin, Syed	3,274,318	3,584,120	2,126,460		241,531	107,481	9,333,910
Murphy, Richard	2,157,652	39,963			175,919	78,284	2,451,818
Murray, Thomas	3,819,895	32,811	358,403		1,749,903	778,707	6,739,719
Nairn, Roderick		1,087,647	116,450		551,432	245,387	2,000,916
Nawaz, Zafar	1,300,238		200,000		157,378	70,033	1,727,649
O'Brien, Richard	22,000	40,000			617,342	274,717	954,059
Oldenburg, Peter	714,028		60,935		450,000	200,250	1,425,213
Pisarri, Thomas	268,830	10,000			211,356	94,053	584,239
Recker, Robert	29,906,146	1,746,646	10,500		3,175,457	1,413,078	36,251,827
Roche, Victoria	59,215				19,435	8,649	87,299
Smith, Derek	352,941			5,000	775,201	344,964	1,478,106
Swanson, Patrick	4,090,518	237,481	1,246,774		640,000	284,800	6,499,573
Ternent, John					14,650	6,519	21,169
Terry, John		10,000			15,000	6,675	31,675
Townley, Robert	6,132,643	1,035,607			19,845	8,831	7,196,926
Tu, Yaping	4,342,923	20,000	218,538		940,000	418,300	5,939,761
Vanderhoof, Jon					19,170	8,531	27,701
Vollberg, Thomas	160,000				150,911	67,155	378,066
Wang, Zhaoyi	2,927,212	20,000	500,000		1,270,000	565,150	5,282,362
Watson, Patrice	303,561				44,058	19,606	367,225
Xiao, Gary	133,279		2,072,180		158,017	70,318	2,433,794
Xiao, Peng	64,575		473,719		213,000	94,785	846,079
Yan, Lin	146,896	34,595			66,568	29,623	277,682
Yee, John	34,595	10,000	96,378		16,106	7,167	164,246
Totals	**$144,460,542**	**$23,593,067**	**$14,021,821**	**$60,000**	**28,910,340**	**$12,865,101**	**$223,910,871**

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

ADMINISTRATION AND PLANNING PROGRAM
Thomas F. Murray, PhD, Principal Investigator

Administration and Planning Program Progress Report

Thomas F. Murray, PhD, Associate Vice Provost for Research and Scholarship, Associate Dean for Research, and Chair of the Department of Pharmacology, serves as the Principal Investigator (PI) of Creighton University's Cancer and Smoking Disease Research Program. Dr. Murray became the PI for the LB595 program at Creighton University on July 1, 2008. He has overall authority and responsibility for the direction and oversight of the program. Dr. Murray seeks and responds to input from the Executive, Internal Advisory, and External Advisory Committees, as well as from the Financial and Compliance Administrator. He ensures that the emphasis at Creighton University continues to be on the development of strong research programs that specialize in particular aspects of cancer and smoking diseases. Dr. Murray provides leadership for planning, implementing, and evaluating such programmatic development and communicates with the State of Nebraska and the appointed external reviewers.

Dr. Murray leads the Administration and Planning Program and the Development Program, and provides oversight of the three Research Program projects. He receives guidance and input from the Executive, External, and Internal Advisory Committees. Beth Herr, Director of Sponsored Programs Administration, provides financial and compliance guidance for the Cancer and Smoking Disease Research Program at Creighton University.

Rev. Timothy R. Lannon, SJ
President, Creighton University (through 1/20/2015)

J. Christopher Bradberry, PharmD
Interim President, Creighton University (through 6/30/2015)

Edward O'Connor, PhD
Provost

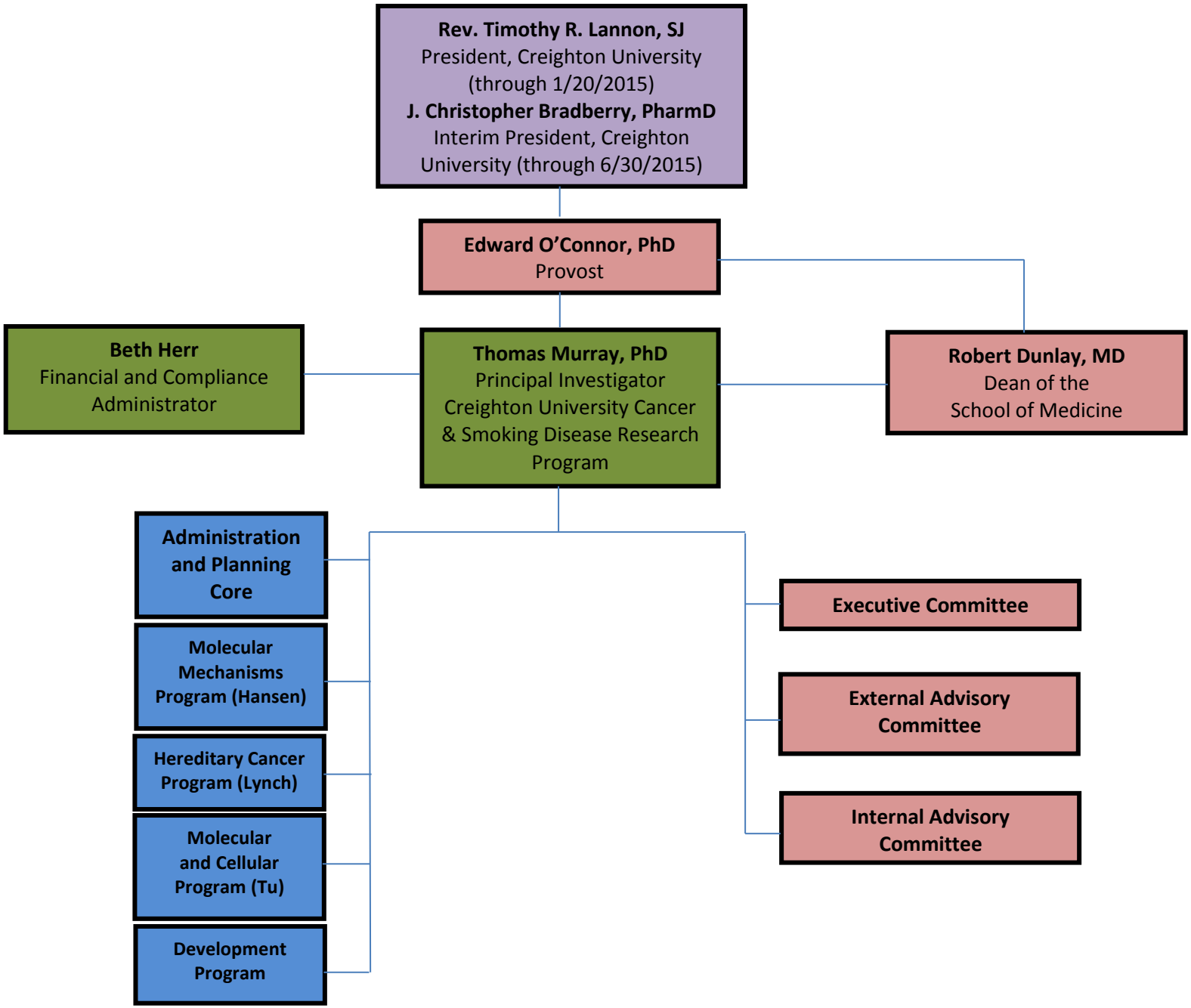
Thomas F. Murray, PhD
Associate Vice Provost for Research & Scholarship Associate Dean for Research, School of Medicine
Principal Investigator, Cancer and Smoking Disease Research Program

Beth Herr
Director, Sponsored Programs Administration; Financial and Compliance Administrator, Cancer and Smoking Disease Research Program

1. **Cancer and Smoking Disease Research Program Administrative Structure**

See the charts to the right and on the following page.

Creighton University Cancer and Smoking Disease Research Program Administrative Structure



The Executive Committee is responsible for overseeing and monitoring the Cancer and Smoking Disease Research Program at Creighton University. It receives all reports from the External Advisory Committee, minutes from all Internal Advisory Committee meetings, reports from the Program Directors, and updates on Development activities. The committee meets on an as-needed basis to assist the Principal Investigator with administrative decisions and to make recommendations regarding programmatic, financial, and compliance issues.

EXECUTIVE COMMITTEE

Thomas F. Murray, PhD
Associate Vice Provost for Research & Scholarship Associate Dean for Research, School of Medicine Chair, Department of Pharmacology
Principal Investigator, Cancer and Smoking Disease Research Program

Robert Dunlay, MD
Dean, School of Medicine

Beth Herr
Director, Sponsored Programs Administration
Financial and Compliance Administrator

2. Internal Advisory Committee

The Internal Advisory Committee reviews all program updates, as well as all committee and state reports. This committee assists with the implementation of recommendations from the State of Nebraska and the External Advisory Committee.

Members of the Internal Advisory Committee for Year 21 are the following:

- Richard Goering, PhD (Chair), Professor and Chair, Department of Medical Microbiology & Immunology, Creighton University School of Medicine
- Anthony Kincaid, PhD (Vice Chair), Professor of Pharmacy Sciences, Creighton University School of Pharmacy and Health Professions
- Thomas F. Murray, PhD, Associate Vice Provost for Research and Scholarship, Associate Dean for Research, Professor and Chair, Department of Pharmacology, Creighton University School of Medicine
- J. Chris Bradberry, PharmD, Dean of Creighton University School of Pharmacy and Health Professions
- Jack Yee, PhD, Professor and Chair, Department of Biomedical Sciences, Creighton University School of Medicine
- Stephen Lanspa, MD, Senior Associate Dean, Professor of Medicine, Division of Gastroenterology, Creighton University School of Medicine

Ex officio members of the Internal Advisory Committee are:

- Beth Herr, Director, Sponsored Programs Administration
- Henry Lynch, MD, Professor and Chair of Preventive Medicine; President, Creighton University Hereditary Cancer Center; Professor of Medicine, Creighton University School of Medicine
- Laura Hansen, PhD, Associate Professor of Biomedical Sciences, Creighton University School of Medicine
- Yaping Tu, PhD, Professor of Pharmacology, Creighton University School of Medicine

3. External Advisory Committee

The External Advisory Committee assists the Principal Investigator with the annual on-site review of the Cancer and Smoking Disease Research Program at Creighton University and with review of applications for the Development Program. Kurt Ebner, PhD, chairs the External Advisory Committee and participates in the State of Nebraska site visit. Additionally, Dr. Ebner provides guidance on an as-needed basis. During the 2014-2015 program year, Dr. Reynold Panettieri, Professor of Medicine at the University of Pennsylvania Medical Center accepted the invitation to join the committee. The committee ensures the implementation of the State of the Nebraska recommendations. The on-site review for the Cancer and Smoking Disease Research Program Year 21 took place at Creighton University on August 30-31, 2015.

Members of the External Advisory Committee are as follows:

- Kurt Ebner, PhD (Chair): University of Kansas Medical Center–Emeritus
- George Bosl, MD: Memorial Sloan-Kettering Cancer Institute
- James P. Calvet, PhD: University of Kansas Medical Center
- Ralf Krahe, PhD: University of Texas MD Anderson Cancer Center
- Donald Miller, MD, PhD: University of Louisville School of Medicine and James Graham Brown Cancer Center
- Reynold Panettieri, Jr., MD: University of Pennsylvania Medical Center
- Richard Robbins, MD, FACP: Methodist Hospital, Houston, Texas

4. Seminars

During Year 21, support was again used to continue an expanded seminar series focused on cancer and smoking-related diseases. This program was directed by Dr. Laura Hansen. Financial support was used to bring in two external speakers with outstanding research expertise in the areas of cancer and smoking-related diseases to give seminars at Creighton University. Scientists from premier institutions who are leaders in their fields were invited to present their cutting-edge research. The seminars were advertised as widely as possible at Creighton, UNMC, and UNL. The seminar series provides opportunities for CU faculty and trainees to meet the speakers, discuss their research, and establish or strengthen collaborations, which will enrich the research environment at CU by facilitating interactions between CU SOM research faculty members and other scientists around the country and stimulate the progress of research projects supported by the LB595 program.

Following is a listing of the speakers and seminar topics for year 21:

- Elliott M. Ross, PhD, Greer Garson and E.E. Fogelson Distinguished Chair in Medical Research, Department of Pharmacology, Green Center for Systems Biology, University of Texas Southwestern Medical Center
  Seminar Topic: Speed and Amplitude in G Protein Signaling Modules
- Adam Karpf, PhD, Associate Professor, University of Nebraska Medical Center

Eppley Institute for Research in Cancer and Allied Diseases
Seminar Topic: DNA Hypomethylation in Epithelial Ovarian Cancer: Pathology and Therapeutic Opportunity

5. Cancer Journal Access at Library

During year 21, funds were used to provide access to the electronic full-text content of relevant cancer research journals. These journals include titles such as the *Journal of the National Cancer Institute* and *Current Opinion in Oncology.* Usage statistics continue to rise as more investigators access the electronic content of these journals.

Creighton University Cancer and Smoking Disease Research Program LB595
External Advisory Committee Site Visit Report

August 30 and 31, 2015

Members of the External Advisory attending were: James P. Calvet PhD, University of Kansas Medical Center; Kurt E. Ebner PhD, Chair of the External Advisory Committee, University of Kansas Medical Center; Donald M. Miller, MD, PhD, University of Louisville; and Reynold Pannettieri, MD, University of Pennsylvania Medical Center. Dr. Pannetteri is a new member of the committee and has expertise in airway biology. Dr. E. O'Connor, Provost, was present at the evening session on August 30.

The purpose of the meeting was to review the Year 21 Progress Report covering July 1, 2014, to June 30, 2015. Attending the scientific sessions were members of the External Advisory Committee; Thomas Murray, PhD, Director of the LB 595 Program at Creighton; Michael White, MD, representing the Internal Advisory Committee; Beth Herr, Sponsored Programs Administration. In addition, Monica Pribil from the Nebraska Department of Health and Human Services attended; she is the new administrator of both the LB506 and LB595 Programs.

Administration and Planning

Thomas Murray, PhD, Principal Investigator of the LB595 Program at Creighton, reviewed the recent administrative changes at Creighton. Creighton is now on a Provost system and Dr. Murray reports directly to the Provost, Dr. Ed O'Connor. External funding at Creighton has remained steady, which is laudatory and has provided about a fivefold return on investment. Based on NIH funding for 2014 on a per faculty basis, Creighton leads the Catholic medical schools, of which there are four. Creighton also has a new internal research fund, the Health Science Strategic Investment Research Fund Faculty Development Grants, which is university-wide. In part, this will replace the former Health Futures Fund. Dr. Murray has provided sterling leadership to the LB595 Program since 2008. This has provided a high degree of stability to the program and has increased the efficiency of the operation with increased external funding.

Development Program

Dr. Murray oversees the development program, which provides funding for new projects by the faculty. The funding is for two years, provided sufficient progress is made after the first year. The intent is to generate data to be used in an application for external funding. Historically, this program has been quite successful in obtaining new external funding.

Four investigators completed their Development Awards and submitted final reports. Patrick Swanson, PhD (Role of Prolactin 2a1 in B Cell Development and Leukemogenesis) has submitted an application to NIH and also submitted a manuscript for publication. He has made significant progress and should be encouraged to apply for

internal funding in order to do further studies so that it is competitive for external funding. Laura Hansen, PhD (Oral Carcinogenesis and ADAM12) has made good progress and also should apply for internal funding to continue the research so that it is competitive for external funding. One of the other programs was terminated after year one and the other one has made some progress in a difficult area.

Applications for new development awards were reviewed by members of the External Advisory Committee and two awards, each for two years, were made starting July, 1 2015. The awards were to: D. Yilmazer-Hanke, PhD (Regulation of 1H1H3 by Nicotine and Tobacco Smoke through the CD 44 Receptor) and to Venkatesh Govindarajan, PhD (Acquired Resistance to Targeted Therapy in Mucinous Colorectal Cancers). They will be reviewed for progress next year.

Seminar Program

There were some concerns about the seminar program since only two speakers made presentations. It appears that the program is fading and should be resurrected. Perhaps some reorganization is in order and it should involve a number of faculty in the LB595 program. Having more speakers could potentially benefit the LB595 program by increasing its visibility, learning from experts to generate new ideas, and forming potential collaborations.

Discovery of Novel Mutations in Hereditary Cancer: Henry Lynch, MD, Program Director

Dr. Lynch's program continues to flourish as evidenced by numerous publications on a continuing basis and the many collaborators from many different institutions. The upcoming symposium in Omaha will provide well-deserved recognition for Dr. Lynch and his pioneering efforts in hereditary cancers. As usual, succession planning is an ongoing issue and its importance must be emphasized; as stated by the Provost, this is a very high priority.

Resource Infrastructure

The resource infrastructure is impressive and is unique to biology. Mr. Dave Van Dyke provides the programming and strategy. He reviewed the continuing efforts to update and secure the database and its repository. A laudatory goal is to make it user-friendly to investigators when they access the database. Recent efforts have been made in developing a specimen barcoding system, document development, and automatic pedigree drawing. Mr. Mark Stacey is responsible for the daily operations and interacts well with investigators who are making requests.

Hematologic Cancers

Dr. Lynch reviewed the progress made by the myeloma group using whole genome and exome sequencing. The biological materials come from the Hereditary Cancer Repository at Creighton. The group continues to search for new mutations in members of families with multiple myeloma and chronic lymphocytic anemia.

BRCAx Breast Cancer
Dr. Lynch reviewed the progress in this area. Individuals of families who have breast cancer but do not have the BRCA mutations are classified as BRCAx. The focus of this research is to discover unknown mutations in order to define the risk to family members. This is a family-specific approach and to-date a family-specific mutation in a BRCAx family was identified as KAT6B. Further studies are in progress with other families.

Molecular Mechanisms and Novel Targets In Cancer: Laura Hansen, Ph.D, Director

Novel Biomarkers and Therapeutic Targets for Nonmelanoma Skin Cancer: L. Hansen, PhD
Significant progress has been made in Aim 1 in determining how 14-3-3 and CDC25A relocalization suppresses squamous cell cancer. CDC25A binds with cytoplasmic 14-3-3 to inhibit apoptosis, but the suppression is not dependent on Cdk1 and is partially dependent through the inhibition of JNK and p38. Further studies are planned. Limited progress was made in Aim 2, which focused on developing a genetic model for targeting 14-3-3c interactions against skin carcinogenesis. They will import a Ywhae mouse to facilitate these studies. Aim 3 is to develop novel peptide inhibitors to 14-3-3c. Initial studies using docking simulations suggest a number of potential candidates to the binding sites on 14-3-3c. There is some concern that these studies may not lead to a breakthrough. The studies may be looking at side effects of increased tumor growth rather than looking at the drivers; to date there does not appear to be a clear connection between the oncogenic mutations and this pathway. Dr. Hansen should consider looking for more compelling pathways to investigate. Dr. Hansen reports four publications based on support from LB 595.

Targeting Androgen Receptor and TRAIL: A Novel Treatment Paradigm for Breast Cancer: Y. Tu, PhD
Dr. Tu has vigorously pursued the studies proposed in Aim 1, assessing the importance of upregulation in TRAIL-resistant breast cancer, and has focused on triple negative breast cancer. Androgen receptor (AR) is a modulator of TRAIL activity and is upregulated in certain breast cancers and correlates with TRAIL resistance. An AR inhibitor enhances the effectiveness of TRAIL and a knockdown of AR upregulates the Death Receptor, DR5, as does inhibition of AR. They have identified a minimal promotor region for the AR inhibitor stimulation of the DR5 gene transcript. Dr. Tu has submitted grant proposals based on this work and lists four publications.

Novel Biomarkers and Potential Therapeutic Targets for Chronic Lymphocytic Leukemia (CLL): Patrick Swanson, PhD
Limited progress was made in Aim 1, where they showed that S100A6 (calcylin) was upregulated in human CLL cells and could interact with annexin. Efforts will continue to find binding partners. Very little effort was made in Aims 2 and 3. The major focus was on Aim 4, which was to complete a previous study. The studies on Aim 4 were focused on the requirement of CD1d and others on the development of CD5+cells and

hypogammaglobulinemia in dnRAG1 and DTG mice. These studies are described in a manuscript under review and there is potential for NIH funding. Unfortunately, there is no data in this report regarding Aim 4; it is recommended that pertinent data be included in this report prior to the submission of the Y 21 progress report to the State. Dr. Swanson has developed an active research program and is vigorously pursuing external funding, which is a central purpose of the LB595 program.

LincRNAs and Oncogenic Activation of NF-kB in Prostate Cancer Progression: Xian-Ming Chen, MD

In prostate cancers, androgen depravation tends to fail as a treatment and subsequently the cancer progresses. Certain lincRNAs may play a role in prostate cancer progression by the activation of NFkbeta, which downregulates suppression of the metastatic genes. This regulation occurs via the 1KKalpha/lincRNA/PRC2 pathway by facilitating the nuclear translocation of 14-3-3e and 14-3-3 eta. Dr. Chen has provided some data to support this view, namely nuclear accumulation of 1KKalpha and translocation of 14-3-3 delta from the nucleus to the cytoplasm. It has been somewhat difficult to review the progress of this grant since no data were presented in the written report; it is recommended that some pertinent data be in the final report to the State. Dr. Chen has an excellent publication record and has been most successful in obtaining external funding. The progress in this study has been somewhat disappointing.

Molecular and Cellular Mechanisms of Smoking-Related Lung Disease Program: Y. Tu, PhD, Director

P-Rex1 Repression and Early-Life Environmental Cigarette Smoke-Increased Risk of Asthma: Y. Tu, PhD

A series of experiments showed that P-Rex1 functions as a negative regulator of neurite outgrowth and extension. Further studies showed that the cytokine IL-6 enhanced neurite growth via protein kinase C repression of P-Rex1. Some progress was made in parts of Aim 1 and 2; taken together, this has generated a working hypothesis amenable for further studies. Dr. Tu lists four publications and external funding from the NIH and Department of Defense.

Molecular and Cellular Triggers of Early-Life Environmental Cigarette Smoke (ECS)-Related Pulmonary Hypertension (PH): Peter Abel, PhD

The overall aim was to determine whether early-life ECS causes inflammation-related hyperinnervation, and whether sensitization of pulmonary vascular to vascular insults in adulthood would cause enhanced pulmonary vascular constriction leading to pulmonary hypertension. Electric field stimulation in lung slices constricts airways and activates cholinergic nerves. Heart rate variability was measured in normal and PRex1 KO mice and is being evaluated as a measure of autonomic nerve activity. Future studies will include ECS-exposed mice. A smoking machine available was unsatisfactory and a new Buxco instrument was purchased, which will facilitate the studies. A procedure was developed to measure right ventricle pressure as a surrogate for measuring pulmonary arterial in intact animals. Progress has been made with all of the three aims.

Targeting the Protein Kinase G (PKG) Pathway in Early-Life Environmental Cigarette Smoke-Related Lung Disease: Peter Oldenburg, PhD

The committee felt that the data presented were superficial, since these inhibitors are not totally specific and this concern was not addressed. There was also considerable alcohol response data presented, which reflected Dr. Oldenburg's other research interest. The committee in their discussions had serious questions whether this work should continue and the decision was made to terminate the project. No further funding is recommended.

Overview of the Molecular and Cellular Mechanisms of Smoking-Related Lung Disease Program

The strength of the proposal is a unique hypothesis and the desire to meet an unmet need to study neuronal control of pulmonary vascular smooth muscle.

Major concerns exist regarding the lack of any preliminary data using *in vivo* models regarding the physiological and pharmacological relevance of P-Rex1. The reviewers strongly suggest that mice with a deficiency in P-Rex1 be studied in an allergen sensitization and challenge model. These data are critical in directing the *in vivo* and *ex vivo* studies. Potentially, a lack of P-Rex1 could modulate neurogenic inflammatory responses, as well as AHR. Alternatively, if there is no phenotype after allergen exposure, the rationale for the *in vitro* studies could be questioned. In a similar manner, ETS *in vivo* should be studied in the P-Rex KO and WT mice to elicit a specific phenotype before embarking on further *in vitro* studies. Furthermore, there may be value in extending studies into electronic cigarette smoke exposure in mice as an alternative and interesting model.

In some of the presentations, the alternating between airway and vascular smooth muscle studies was confusing. Pulmonary hypertension does not occur in asthma, and secondary pulmonary hypertension in COPD afflicts a minority of patients. There may be greater utility in studying vascular permeability in the airways in the presence and absence of ETS exposure and P-Rex. If the focus is on pulmonary hypertension, the studies should use more conventional models, such as chronic hypoxia or pulmonary emboli.

This group has the potential to ask another important question, which is how can early smoking lead to lasting or permanent effects on airway smooth muscle cells and the vasculature that are seen years later. It seems that nuclear events, such as changes in gene expression, could be the cause, and this could result from heritable epigenetic changes to the genome. This group could explore the possibility of doing RNA sequencing (RNA-seq) to look for global changes in gene expression, and they could explore DNA methylation or protein chromatin modifications, such as methylation, acetylation, phosphorylation etc. There are new tools being generated, including mouse models in which there is conditional knockout of epigenetic regulators. It seems that new information in this area on the epigenetic effects of cigarette smoking would attract attention for future funding. It is recommended that this group consult with others in the field to explore the feasibility of these ideas.

The committee recommends that the funds allocated to Dr.Oldlenburg be transferred to Dr. Tu to be used in fostering the recommendations made by the committee. In addition, Dr. Tu should discuss with his consultants how best to proceed.

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 (July 1, 2014 – June 30, 2015)

DISCOVERY OF NOVEL MUTATIONS IN HEREDITARY CANCER RESEARCH PROGRAM PROGRESS REPORT
Henry Lynch, MD, Director

Project 1: Database
Principal Investigator: Henry Lynch, MD

I. Progress Report Summary

A. Specific Aims
The specific aim is to continue with the ongoing process of database facility upgrade and data quality improvement. We redesigned the database in 2004 and have benefited from increased flexibility and security for our data. During the time since 2004, the major focus of the database manager (Mark Stacey, MS, MBA) and the database consultant (David Van Dyke) has been to further increase the functionality of this database, including staff productivity and database accuracy. Particular areas of focus during the past year are listed in the progress report.

B. Studies and Results

Creighton Preventive Medicine Research Laboratory Progress
During the past year, much of the efforts of Dina Becirovic, BS, were spent on identifying potential vendors for the initiation of a barcoding system in the laboratory. Based on our current practices and future endeavors, the decision was narrowed down to three vendors: GA International, CompuType, and Wheaton. These findings were then presented to Dr. Lynch; Carrie Snyder, MSN, APRN-CNS; Mark Stacey; and David Van Dyke. After extensive research and collaboration, a decision was made to use Wheaton cryovials with 2D barcode inserts, in addition to labeling the sample on the outside of the cryovial prior to storing for additional integrity maintenance. Dina Becirovic and David Van Dyke worked closely on determining the number and types of labels necessary for each type of specimen we store, as well as finding out the best manner in which the labels would be integrated into day-to-day workflow. Two printers, two scanners, one Plurascan, and necessary labels were ordered. In addition, a NanoDrop 8000 was purchased for accurate and more efficient processing of samples. As budgeted, a liquid nitrogen tank was purchased to maintain the integrity and storage of current and new invaluable samples that will be arriving shortly.

During the past year, the Hereditary Cancer Research Center received more than 1,000 stained and unstained slides that had been stored with a past collaborator. These slides and blocks pertain to breast cancer cases from some of our well-defined hereditary breast cancer families. These samples are currently being processed and put into storage and linked to the specific cancer site for the individual in the database for easy retrieval in the future, if needed.

In addition, we were contacted by another longstanding collaborator at the International Agency of Cancer Research in Lyon, France, whose research has taken a different direction. Therefore, the collaborator is planning to ship all research samples that were provided to them since the late 1980s from many of our hereditary breast cancer families to be stored indefinitely in our biorepository for future studies. These samples consist of extracted DNA, snap pellets, and

lymphocytic cell lines. Dina Becirovic, with the assistance of experienced members of the International Society for Biological and Environmental Biorepositories (ISBER), was able to identify the most reliable international shipment couriers to arrange for this shipment of more than 3,000 samples from Lyon without compromising the integrity of the samples. The first shipment is scheduled for August 3, 2015.

The HCC Repository receives and stores a variety of samples, including lymphoblastoid cell lines, fibroblasts, DNA samples, frozen tissue samples, formalin tissue, blocks, slides, saliva, serum, and plasma. The serum and plasma samples are immediately aliquoted in the smaller quantities. The blood samples, after processing, are stored as "DMSO lymphocytes"; untransformed lymphocytes in "DMSO solution" and snap lymphocytes pellets are put in culture to grow lymphoblastoid cell lines. The biological samples are collected from members of families with a history of breast cancer, breast/ovarian cancer, Li-Fraumeni syndrome, colon cancer, juvenile polyposis, multiple myeloma, leukemia, lymphoma, pancreas, and gastric cancer. Creighton's HCC continues to collaborate with various individuals and institutions nationally and internationally, most prominent of which are Steve Lipkin, MD, PhD, Weill Medical College of Cornell University; Megan Hitchens, PhD, Stanford University; San Ming Wang, MD, University of Nebraska Medical Center; Steven Narod, MD, Women's College Hospital, Toronto, Ontario,; Gustavo Mostoslavsky, MD, PhD; Hemant Roy, MD, PhD, of Boston University and Boston University Medical Center; Ralf Krahe, PhD, University of Texas MD Anderson Cancer Center; and Isabel Spier, MD, University of Bonn, Germany. Our HCC continues to supply DNA and transformed and untransformed lymphocytes samples. The HCC's most dominant project, aside from sample processing, is currently the growth of cell lines on several hematological and *BRCA*-positive families, including family Nos. 4493, 4671, 5066 and 5756. Other projects include cell line growth for San Ming Wang, MD, along with DNA extraction and supply. The HCC continuously looks at recognizing the future need of our samples and making DNA available for research at the time of the initial request.

The purpose of the Biosamples Repository is to:

- Provide support services for the genetic studies, including biosamples collection and distribution;
- Establish and develop lymphoblastoid cell lines;
- Create large-scale culture of cell lines for DNA/RNA;
- Establish fibroblast cell lines;
- Extract DNA and routinely analyze molecular genetics;
- Analyze the data related to the samples that have been collected for ongoing and new studies;
- Gather and store the samples from studies that have been completed;
- Rapidly process, appropriately label, and aliquot samples;
- Ship biosamples to designated facilities and collaborators for testing;
- Store biosamples for the long term (+20 years); and
- Monitor the locations and data of the specimens being received, stored, or shipped to researchers/collaborators.

Particular areas of database effort during year 21:

- The major and significant accomplishment of a recent prior period (year 17) was to complete the migration of the previous Microsoft Access database to SQL Server, a more robust platform. The database has been completely functioning in the SQL Server environment during year 21 and has been working as expected since its conversion.

We continue to use Microsoft Access as our user interface into the SQL Server database, but the data itself resides on the SQL Server. During years 18-21, we have rebuilt, enhanced, and added special working modules used to query the database. These modules remain in Microsoft Access and must be compatible with a special translator (known as an ODBC connection) used to retrieve information for the queries from the SQL Server platform.

- Significant progress has been made in the development of an automated pedigree drawing program. This involves leveraging of the existing pedigree schema, which exists in a table (non-graphical) format, into a data structure that is linked to a graphic presentation program. Other attributes, such as age, gender, cancers, etc., are incorporated into this data structure. The program is now capable of drawing most family structures, and development continues to broaden its capability.
- Work is underway to convert lab specimen storage to a barcoding system. A vial labeling system has been selected and a printer purchased. Development of a database program code for this process is in progress.
- We further expanded the data integrity warning system. This procedure, run periodically, produces a report that highlights illogical/invalid data entry values so that the resource associates can check and clean up as needed. More data checks were added to the report during year 21, as were more data displays to facilitate locating problems by family mutation or error type. The report has been supplemented with one-time cleanup items found in connection with research activities. We added a proband error check to the database family report.
- The lab data quality report was run several times during the year. This report performs 14 quality checks on lab records in the database and lists any problems.
- Additional family groups were evaluated for out-of-date risk coding, identifying cases where risk routines need to be re-run for the family.
- Work continued on reusable queries for organizing data in formats required by specific multicenter studies, including recoding and checks for completeness and consistency. More was done with program control to implement more complex logic and reduce manual efforts. The checking process also enhances the quality of analysis data sets.
- An auxiliary database model was developed to calculate cancer syndrome and genetic risk retroactively at each point in time when there was a change for a family. This is used for studies utilizing time series information from the database.
- We implemented the “famCheck” auxiliary database, which monitors progress with data input for new families and updated family information.
- Work is continuing on a more comprehensive database manual that addresses issues of research and technical interests. This manual will be augmented and updated on a continuing basis going forward.

In all these efforts, feedback from the departmental staff has been crucial in guiding the efforts of database management.

C. Significance

The migration of the database was aimed at maintaining previously developed functionality, while increasing our flexibility in storing information, increasing the richness of that information, and allowing more responsiveness to the changes in research needs on a new SQL Server platform. Data quality assessments and improvements were made at each step. Achievement of our goals has given us an increasingly effective and secure facility for data storage and manipulation; increasingly efficient facilities for identifying observations for inclusion in new collaborative and in-house studies; and increasingly accurate data to use in these studies.

D. Plans

We plan to continue working on enhancements and quality improvements listed above (Results section), and deal with new issues that will arise during the course of the coming year. In addition, we plan on implementing a pedigree drawing program that will remove our dependence on the manual pedigree drawing process currently in place. A barcoding system for lab specimen is in progress. Database enhancements to translate between barcodes and specimen IDs are required. Further documentation will be added to the database manual, which will facilitate new users' understanding and analysis of the data in the existing system.

E. Database Statistics

The following table summarizes a subset of information currently in the database, with emphasis on five family groups highly targeted for collaborative studies. The number of families is shown, as well as the number of individuals in those families (column Indiv), and the number of individuals with DNA or lymphocytes in storage (column Spec). The number of individuals who have tested positive or negative for a mutation is also included.

Database Overview

		Positive		Negative		Total Bloodline		Total
Family Group	Families	Indiv	Spec	Indiv	Spec	Indiv	Spec	Indiv
BRCA Mutation-women w/cancer		435	339	71	48	1,393	394	
BRCA Mutation-women w/o cancer		292	245	749	688	7,035	991	
BRCA Mutation-men w/cancer		21	20	17	17	154	38	
BRCA Mutation-men w/o cancer		259	245	345	324	7,571	605	
Total BRCA Mutation families	195	1,007	849	1,182	1,077	16,153	2,028	31,589
Neg BRCA screening in family	231					15,150	749	24,815
Other Breast families	439					39,537	193	40,839
Total Breast Families	865					70,840	2,970	97,243
MMR Mutation-indiv w/ cancer		282	249	24	23	966	304	
MMR Mutation-indiv w/o cancer		290	269	739	698	10,426	1,031	
Total MMR Mutation families	101	572	518	763	721	11,392	1,335	19,086
Neg MMR screening in family	16					1,288	112	2,124
Other Lynch Syndrome families	240					14,138	204	19,991
Total Lynch Syndrome Families	357					26,818	1,651	41,201
APC/MYH Mutation-indiv w/ cancer		27	20	0	0	59	20	
APC/MYH Mutation-indiv w/o cancer		65	50	59	43	562	94	
Total APC/MYH Mutation families	12	92	70	59	43	621	114	996
Neg APC/MYH screening in family	5					940	72	1,390
Other FAP families	23					1,233	18	1,376
Total FAP Families	40					2,794	204	3,762

CDKN2A Mutation-indiv w/ cancer		23	19	3	3	70	22	
CDKN2A Mutation-indiv w/o cancer		16	15	55	53	773	69	
Total CDKN2A Mutation families	10	39	34	58	56	843	91	1,039
Neg CDKN2A screening in family	7					400	10	519
Other Melanoma families	83					9,574	752	14,554
Total Melanoma Families	100					10,817	853	16,112
MMyeloma -indiv w/ cancer						166	50	
MMyeloma -indiv w/o cancer						3,322	203	
Total Multiple Myeloma Famlies	69					3,488	253	6,189
Other Research Families	950							46,246
Total Research Families	2,301							189,583
Total Database	3,300							221,963

Cancers included above include those of interest for the syndrome, such as breast and ovarian with the BRCA mutations. In addition to DNA and lymphocytes, the database contains storage and tracking information on other lab specimen (e.g., serum, whole blood).

During year 21, 10 families with 299 individuals were added to the database. Genetic test results were reported for 47 individuals, whole blood was received in the lab for 60 individuals, 295 components were created, and 517 specimen were sent to collaborators, all of which was tracked by the database.

II. List of publications (7/1/2014 – 6/30/2015)
List of refereed publications germane to LB595 funding from July 1, 2014 – June 30, 2015.
[Note: An asterisk is placed at the beginning of each publication or grant directly related to LB595, followed by the related component number. Although Component I affects nearly every study we are involved in, we have designated as “directly related” some in which the database updating has been especially instrumental.]

1. *I and III. Wen H, Kim YC, Snyder C, Xiao F, Fleissner EA, Becirovic D, Luo J, Downs B, Sherman S, Cowan KH, Lynch HT, Wang SM. Family-specific, novel, deleterious germline variants provide a rich resource to identify genetic predispositions for BRCAx familial breast cancer. *BMC Cancer* 2014;14:470.

2. *I. Park DJ, Tao K, Le Calvez-Kelm F, Nguyen-Dumont T, Robinot N, Hammet F, Odefrey F, Tsimiklis H, Teo ZL, Thingholm LB, Young EL, Voegele C, Lonie A, Pope BJ, Roane TC, Bell R, Hu H, Shankaracharya, Huff CD, Ellis J, Li J, Makunin IV, John EM, Andrulis IL, Terry MB, Daly M, Buys SS, Snyder C, Lynch HT, Devilee P, Giles GG, Hopper JL, Feng BJ, Lesueur F, Tavtigian SV, Southey MC, Goldgar DE. Rare mutations in RINT1 predispose carriers to breast and Lynch syndrome-spectrum cancers. *Cancer Discov* 2014;4:804-815.

3. *I. Giannakeas V, Lubinski J, Gronwald J, Moller P, Armel S, Lynch HT, Foulkes WD, Kim-Sing C, Singer C, Neuhausen SL, Friedman E, Tung N, Senter L, Sun P, Narod SA.

Mammography screening and the risk of breast cancer in BRCA1 and BRCA2 mutation carriers: a prospective study. *Breast Cancer Res Treat* 2014;147:113-118.

4. Lynch HT, Drescher K, Knezetic J, Lanspa S. Genetics, biomarkers, hereditary cancer syndrome diagnosis, heterogeneity and treatment: a review. *Curr Treat Options Oncol* 2014;15:429-442.

5. Schlussel AT, Gagliano RA Jr, Seto-Donlon S, Eggerding F, Donlon T, Berenberg J, Lynch HT. The evolution of colorectal cancer genetics-Part 1: from discovery to practice. *J Gastrointest Oncol* 2014;5:326-335.

6. Schlussel AT, Gagliano RA Jr, Seto-Donlon S, Eggerding F, Donlon T, Berenberg J, Lynch HT. The evolution of colorectal cancer genetics-Part 2: clinical implications and applications. *J Gastrointest Oncol* 2014;5:336-344.

7. *I. Chai X, Friebel TM, singer CF, Evans DG, Lynch HT, Isaacs C, Garber JE, Neuhausen SL, Matloff E, Eeles R, Tung N, Weitzel JN, Couch FJ, Hulick PJ, Ganz PA, Daly MB, Olopade OI, Tomlinson G, Blum JL, Domchek SM, Chen J, Rebbeck TR. Use of risk-reducing surgeries in a prospective cohort of 1,499 BRCA1 and BRCA2 mutation carriers. *Breast Cancer Res Treat* 2014;148:397-406.

8. Gatalica Z, Snyder C, Maney T, Ghazalpour A, Holterman DA, Xiao N, Overberg P, Rose I, Basu GD, Vranic S, Lynch HT, Von Hoff DD, Hamid O. Programmed cell death 1 (PD-1) and its ligand (PD-L1) in common cancers and their correlation with molecular cancer type. *Cancer Epidemiol Biomarkers Prev* 2014;23:2965-2970.

9. Lanspa SJ, Lynch HT. Sessile serrated adenomas: why conventional endoscopy is okay for unconventional polyps. *Dig Dis Sci* 2014;59:2848-2849.

10. *III. Lynch H, Snyder C, Wang SM. Considerations for comprehensive assessment of genetic predisposition in familial breast cancer. *Breast J* 2015;21:67-75.

11. Bikhchandani J, Lynch HT. Commentary on 'Colonoscopy screening compliance and outcomes in patients with Lynch syndrome'. *Colorectal Dis* 2015;17:46-49.

12. Eskander RN, Lynch HT, Brown SM, Wagman LD, Tewari KS. Novel MSH2 mutation in the first report of a Vietnamese-American kindred with Lynch syndrome. *Gynecol Oncol Rep* 2015;12:31-33.

13. Lynch HT, Snyder CL, Shaw TG, Heinen CD, Hitchins MP. Milestones of Lynch syndrome: 1895-2015. *Nat Rev Cancer* 2015;15:181-194.

14. Hansford S, Kaurah P, Li-chang H, Woo M, Senz J, Pinheiro H, Schrader KA, Schaeffer DF, Shumansky K, Zogopoulos G, Santos TA, Claro I, Carvalho J, Nielsen C, Padilla S, Lum A, Talhouk A, Baker-Lange K, Richardson S, Lewis I, Lindor NM, Pennell E, MacMillan A, Fernandez B, keller G, Lynch H, Shah sP, Guilford P, Gallinger S, Corso G, Roviello F, Caldas C, Oliveira C, Pharoah PD, Huntsman DG. Hereditary diffuse gastric cancer syndrome: CDH1 mutations and beyond. *JAMA Oncol* 2015;1:306-313.

15. *I. Snyder C, Metcalfe K, Sopik V, Royer R, Zhang S, Narod SA, Akbari MR, Lynch HT. Prevalence of PALB2 mutations in the Creighton University Breast Cancer Family Registry. *Breast Cancer Res Treat* 2015;150:637-641.

16. Lynch HT, Lanspa SJ, Snyder CL, Drescher KM. Microsatellite instability, disease-free survival and role of tumour infiltrating lymphocytes (Invited editorial on 'Predictors of disease-free survival in colorectal cancer with microsatellite instability: An AGEO multicenter study'). *Eur J Cancer* 2015;51:922-924.

17. *I and III. Downs B, Kim YC, Xiao F, Snyder C, Chen P, Fleissner EA, Becirovic D, Wen H, Sherman S, Cowan KH, Lynch HT, Wang SM. Two PALB2 germline mutations found in both BRCA1+ and BRCAx familial breast cancer. *Breast Cancer Res Treat* 2015;151:219-224.

18. *I. Casey MJ, Bewtra C, Lynch HT, Snyder CL, Stacey M. Endometrial cancers in mutation carriers from hereditary breast ovarian cancer syndrome kindreds: report from the Creighton University Hereditary Cancer Registry with review of the implications. *Int J Gynecol Cancer* 2015;25:650-656.

19. *I. Cybulski C, Lubinski J, Huzarski T, Lynch HT, Randall SA, Neuhausen SL, Senter L, Friedman S, Ainsworth P, Singer C, Foulkes WD, Narod SA, Sun P, Kotsopoulos J. Prospective evaluation of alcohol consumption and the risk of breast cancer in BRCA1 and BRCA2 mutation carriers. *Breast Cancer Res Treat* 2015;151:435-441.

20. *I. Metcalfe K, Lynch HT, Foulkes WD, Tung N, Kim-Sing C, Olopade OI, Eisen A, Rosen B, Snyder C, Gershman S, Sun P, Narod SA. Effect of oophorectomy on survival after breast cancer in BRCA1 and BRCA2 mutation carriers. *JAMA Oncol* 2015;1:306-313.

21. *I. Segev Y, Rosen B, Lubinski J, Gronwald J, Lynch HT, Moller P, Kim-Sing C, Ghadirian P, Karlan B, Eng C, Gilchrist D, Neuhausen SL, Eisen A, Friedman E, Euhus D, Ping S, Narod SA; Hereditary Breast Cancer Study Group. Risk factors for endometrial cancer among women with a BRCA1 or BRCA2 mutation: a case control study. *Fam Cancer*. In press.

22. Goodenberger ML, Thomas BC, Riegert-Johnson D, Boland CR, Plon SE, Clendenning M, Win AK, Senter L, Lipkin SM, Stadler ZK, Macrae FA, Lynch HT, Weitzel JN, de la Chapelle A, Syngal S, Lynch P, Parry S, Jenkins MA, Gallinger S, Holter S, Aronson M, Newcomb PA, Burnett T, Le Marchand L, Pichurin P, Hampel H, Terdiman JP, Lu KH, Thibodeau S, Lindor NM. PSM2 monoallelic mutation carriers: the known unknown. *Genet Med*. In press.

23. Tiwari AK, Roy HK, Lynch HT. Lynch syndrome in the 21st century: clinical perspectives. *QJM*. In press.

III. List of extramural grants submitted from 7/1/2014-6/30/2015 but not awarded

*II. National Institutes of Health
Title: Identification of genetic factors for familial lymphoid cancers
PI: Henry Lynch

Total funds requested: $108,796

*III. National Institutes of Health
Title: Genetic predisposition in BRCAx familial breast cancer
PI: Henry Lynch
Total funds requested: $424,731

National Institutes of Health
Title: Evolution and somatic landscape of Lynch syndrome-associated tumors
PI: Henry Lynch
Total funds requested: $297,855

*III. National Institutes of Health
Title: Predisposition in XRCC5 promoter contributing to familial breast cancer
PI: Henry Lynch
Total funds requested: $40,000

*III. National Institutes of Health
Title: Early genome instability in BRCA1+ familial breast cancer
PI: Henry Lynch
Total funds requested: $60,000

National Institutes of Health
Title: Predictive testing and screening uptake in Lynch syndrome: electronic education vs. usual care
PI: Henry Lynch
Total funds requested: $400,125

IV. List of extramural grants awarded from 7/1/2014-6/30/2015

*II. National Institutes of Health
Title: Whole genome sequencing to discover familial myeloma risk genes
PI: Henry Lynch
Total funds awarded: $59,607

*III. National Institutes of Health
Title: PDQ3 genetic basis of breast cancer resistance in BRCA1+ carrier
PI: Henry Lynch
Total funds awarded: $43,500

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 (July 1, 2014 – June 30, 2015)

DISCOVERY OF NOVEL MUTATIONS IN HEREDITARY CANCER RESEARCH PROGRAM PROGRESS REPORT

Henry Lynch, MD, Director

Project 2: Hematologic Cancers

Principal Investigator: Henry T. Lynch, MD

I. Progress Report Summary

A. Specific Aims

Our specific aim remains accruing families with a pattern of familial hematological cancers, namely multiple myeloma and chronic lymphocytic leukemia, and searching for new mutations.

- Aim 1: Continue ongoing research with informative pedigrees in familial chronic lymphocytic leukemia (CLL), multiple myeloma (MM), various lymphomas, and mixed hematologic cancers, with highly skilled collaborators (Stephen Lipkin, MD, PhD; Ken Offit, MD, MPH; Angela-Brooks-Wilson, PhD) to identify novel germline predisposition risk genes.

B. Studies and Results

The majority of hereditary forms of cancer are characterized by phenotypic and genotypic heterogeneity. Our hypothesis is that MM has an association with several hereditary cancer syndromes and, therefore, it is highly likely that cancer-causing mutations in this disorder are heterogeneous.

Reports of substantial familial clustering of MM/MGUS have been reported, including by our own team. The relative risk (RR) of MM in first-degree relatives of probands ranges from 2-4. The risk of hematologic cancers, especially CLL and non-Hodgkin's lymphoma is higher in relatives of MM probands. In the largest study, a cohort of 4458 MGUS/MM patients and 14,621 first degree relatives found an RR of 5.0 for CLL. Increased risk of several solid tumors is also associated with MM, most notably and consistently for prostate cancer (RR ~3-4) and pancreatic cancer (also observed by members of our team). In summary, the Familial Myeloma inheritance pattern is consistent with the existence of specific, highly penetrant risk genes that predispose to Familial Myeloma and associated malignancies, most notably CLL, NHL, and prostate and pancreatic cancer, with an autosomal dominant inheritance pattern. MM paraproteins are derived from IgG, IgM, and IgA light and heavy chains. An appreciable fraction of MM/MGUS paraproteins bind to protein antigens called paratargs (paraprotein targets). These are derived from a variety of different proteins (e.g., paratarg-8 is a fragment of the cytoplasmic autophagy protein ATG13). For unknown reasons, paraprotein paratargs are hyperphosphorylated. The same hyper-phosphorylated paratarg antigen and the same paraprotein immunoglobulin light/heavy chain subclass (e.g., IgG_3) are frequently shared in common by multiple affected individuals in multiple generations of the same Familial Myeloma kindred, and are inherited with an autosomal dominant trait pattern. In Familial Myeloma, the presence of the same paratarg antigen found in affected family members also found in unaffected family members confers an odds ratio of 6.2 for developing MGUS. In sporadic

disease, paratargs are also observed, and the presence in serum of previously identified hyperphosphorylated paratargs increases relative risk of developing MM/MGUS from 7-13 fold. In summary, while the precise mechanistic basis for the presence of hyper-phosphorylated paratarg antigens in serum shared by multiple Familial MM/MGUS family members is poorly understood, their detection in multiple affected and pre-symptomatic unaffected family members from the same kindreds gives potentially important clues to possible candidate genes and signaling pathways for cross comparison with mutations identified by whole exome/genome sequencing to understand the etiology of Familial Myeloma.

Samples from our biorepository collected on our registered familial MM families have been sent to Steve Lipkin, MD, PhD, at Cornell University for whole genome and whole exome sequencing. In addition, several control samples have been sent for use in his analysis of candidate gene mutations identified in our familial MM families. Table 1 provides details numbers of families and samples available for various familial hematological syndromes within the Hereditary Cancer Center registry and biorepository. We continue to collaborate with Geraldine Schechter, MD, and her colleague Anita Aggrawal, MD, at the Washington VA Medical Center, Washington, D.C.

Table 1: Description of Familial Hematologic Families Resource

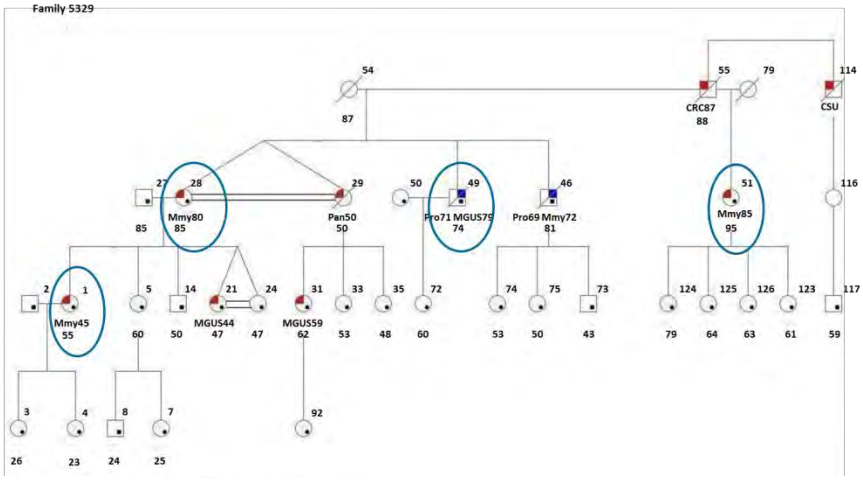
Type	# of Families*	# AFF Samples	# UnAFF Samples	# AFF -NoSample	# UnAFF -NoSample
Familial MM	42	40	129	15	389
Familial CLL	10	12	55	6	158
Familial Leukemia	49	4	88	26	535
Familial Lymphoma	130	46	223	115	1491
Mixed	65	11	28	33	515
Total	276^	101^	418^	189^	2726^

*Have at least 2 of the cancers of interest within the bloodline.
^Some families qualify for more than one type

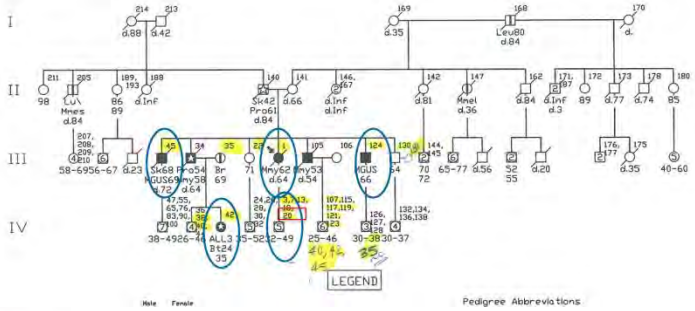
Whole exome and whole genome sequencing of constitutional DNA have successfully identified novel gene mutations in several disorders. However, WES/WGS are limited by the large number of rare variants in each genome that confound causative mutation identification. Here, we are taking advantage of segregation in well-sampled large families and also cross-compare cancer-specific databases of both constitutional and somatic mutations (e.g., Myeloma WGS somatic mutation profiling studies, TCGA constitutional and somatic, and COSMIC) to provide a Mendelian filter to narrow the list of candidates, allowing discovery and validation of mutations in novel Familial Myeloma risk genes.

The focus has primarily been on sequencing multiple affected individuals from different Familial MM kindreds. To date, Lipkin's group has sequenced 11 individual constitutional exomes and 7 constitutional whole genomes.

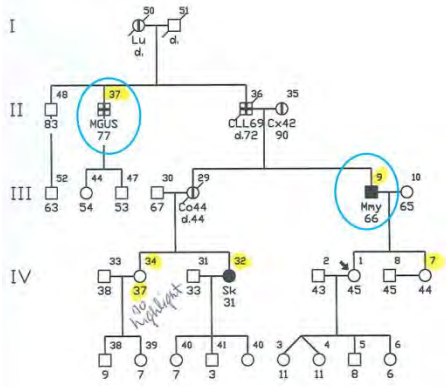
Selected pedigrees are shown below in **Figure 1**.



Family 4493



Family 5066



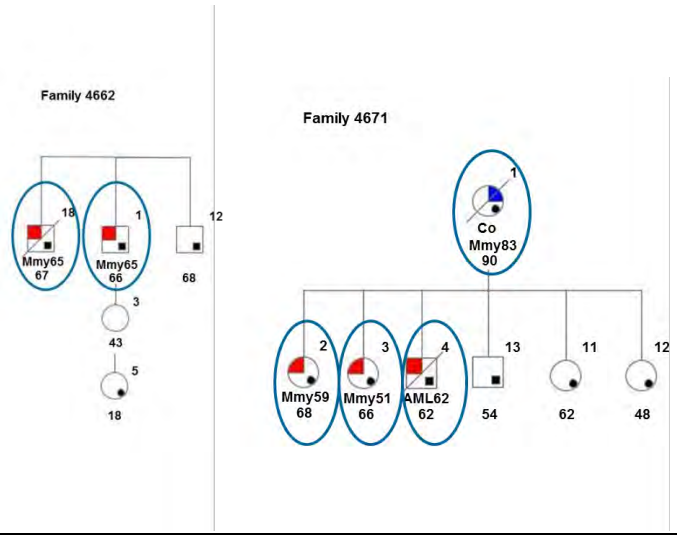


FIGURE 1: Example of a Familial Myeloma family autosomal dominant inheritance pattern. Current age and age of incident cancer are shown for each individual. "**0**", proband sequenced by WES/WGS. Mmy, multiple myeloma, MGUS, monoclonal gammopathy of unknown significance. Pro, prostate cancer. Pan, pancreatic cancer, CRC, colorectal cancer. CSU, cancer site unknown but likely prostate cancer.

Briefly, Lipkin's group has optimized methods for library generation, exome capture, sequencing, data processing, and bioinformatic analyses for the identification of constitutional (germline) variants in DNA from normal (blood) samples. Library construction is carried out using TruSeq reagents, followed by in-solution capture of exomic regions from 20,766 genes using the Agilent SureSelect 50Mb kit.

Libraries are sequenced using Ilumina HiSeq 2000 Genomic Analyzer instruments and the resulting paired-end data aligned to the human reference sequence (hg18) using the ELAND algorithm of CASAVA 1.7 software. The stages involved in subsequent analysis are 1) the identification of mutations (single base substitutions, insertions, deletions, and complex alterations) in the normal sequence compared to the reference sequence; 2) determination of the number of sequences with each mutation; 3) identification of genes harboring mutations and the amino acid consequences of the alterations; and 4) calculation of the coverage (number of times each base is represented at a high quality) across the exome. Filters are then applied to remove remaining false positives. These include requirements for minimum quality, coverage, number of distinct tags and mapping to a single region of the genome. Additionally, the data are visually inspected for any mutations of potential interest.

GATK pipeline variant calling has been run to detect variants that are less well represented by the ELAND assembly.

Further bioinformatics steps to compare mutations to variant databases (dbSNP and ESP) to determine whether the alterations are common polymorphisms or rare or private variants are also carried out (**Figure 2**).

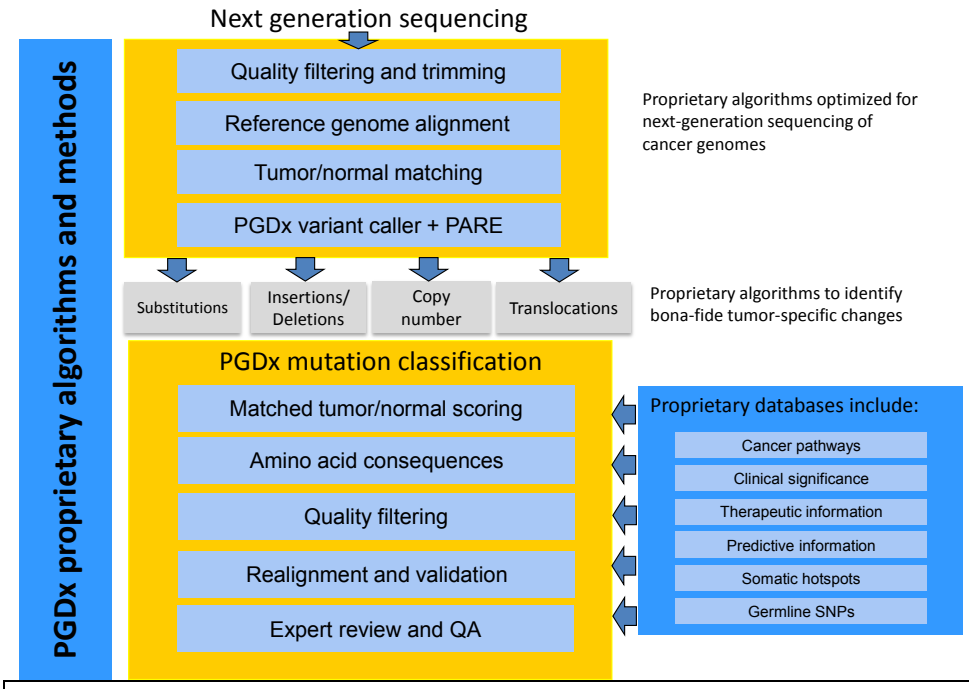


FIGURE 2: Analysis Pipeline for Constitutional Exome and Genome Sequencing

Sanger sequencing selected coding SNVs and structural variants (SVs) for co-segregating candidate variants will be utilized. In addition, Lipkin's group will follow up on gene signaling pathways including TOR signaling, RNA processing, and STAT pathway signaling.

Additionally, the ULK4 variant identified in our first kindred as a co-segregating mutation is being studied further. An additional kindred that has paratarg-8+ myeloma cells (ATG13L1) has been obtained. Sequencing of ULK4 did identify the identical ULK4 D1259G variant or truncating variants. However, the ULK4 non-synonymous SNP was not identified.

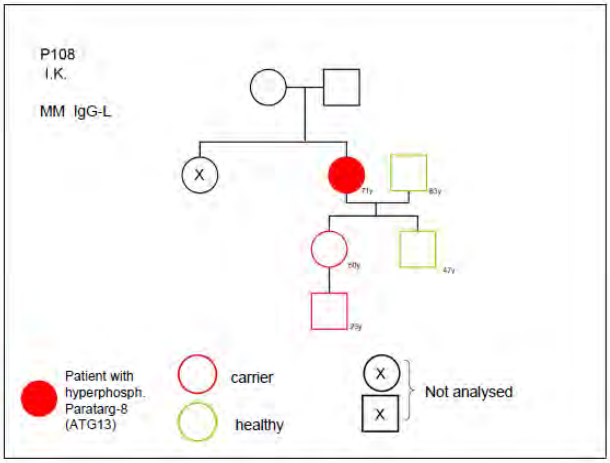


FIGURE 3: Pedigree of New Paratarg-8+ Myeloma Kindred

Next, Lipkin's group is in the process of exome analyses of an additional 16 probands with familial and early-onset disease. They are also performing co-segregation analysis of variants and functional studies of ULK4. Their overall goal is to identify ~80 candidates for TruSeq

validation using a validation cohort of non-overlapping familial and early onset kindreds. They have also been in touch with the Multiple Myeloma Research Foundation (MMRF) to have access to test variants in their computational data of sporadic constitutional MM exome probands.

Additionally, haplotype sharing has been used to evaluate for regions inherited in common in multiple kindreds that co-segregate with the phenotype of MM/MGUS. While no haplotype is shared among all kindreds analyzed to date, we do see sharing of inheritance by descent haplotypes in the TAL1 gene. TAL1 is associated with leukemogenesis and, therefore, becomes a candidate to evaluate for non-coding mutations increasing risk of MGUS and Familial Myeloma.

Collection of families with a familial pattern of lymphoid cancers continues. See Table 1 above. We continue to collaborate with Angela Brooks-Wilson, PhD, in Vancouver, BC. Funding for additional whole genome sequencing is being pursued through pilot studies and the identification of potential candidate genes.

C. Significance
Identification of germline mutations in hematologic cancer kindreds will enable early detection and improved therapy and prevention. CLL is the most common B-cell malignancy with an 8.5-fold increased risk among first-degree relatives. Multiple myeloma accounts for 10-15% of the total hematologic cancer burden with a 2-fold increased risk in first-degree relatives. Lymphoid cancers affect hundreds of thousands worldwide.

II. List of publications (7/1/2014 – 6/30/2015)
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PI: Henry Lynch
Total funds awarded: $43,500

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 (July 1, 2014 – June 30, 2015)

DISCOVERY OF NOVEL MUTATIONS IN HEREDITARY CANCER RESEARCH PROGRAM PROGRESS REPORT

Henry T. Lynch, MD, Director

Project 3: BRCAx Breast Cancer

Principal Investigator: Henry T. Lynch, MD

I. Progress Report Summary

A. Specific Aims

- Aim 1: Ascertain and provide samples of current and newly ascertained BRCAx families in search of exome variants and other molecular genetic findings.
- Aim 2: Describe genotypic-phenotypic correlations of candidate variants with the BRCAx families.

B. Studies and Results

The Creighton Hereditary Cancer Center (HCC) utilizes a family-based approach in the study of hereditary cancers. Currently, our resource has 186 highly extended and cancer-verified BRCAx FBC families, with 7 newly added this past year. Continued development of these families, as well as ascertainment of new families, is essential for us to provide our collaborator with the samples needed to advance research in this area. Genetic samples from 537 individuals within the 186 BRCAx families have been collected and stored; 245 have been affected with breast cancer and 292 are unaffected.

During this past year (Year 21 of our LB595 funding), 188 genetic samples have been processed with DNA extraction completed in our biorepository and provided to Wang and his team for exome sequencing and analysis. Below are some of the findings from Wang's research efforts utilizing our samples and BRCAX families.

Our research with Dr. Wang at UNMC and his highly trained laboratory colleagues on BRCAx FBC families has identified perhaps the first example of a family-specific mutation in a BRCAx family[16] through exome sequencing. See Figure 1 below. This mutation (KAT6B) was identified exclusively in this family, which was negative for *BRCA1* and *BRCA2* mutations. Four of the five women affected with breast cancer were identified as carriers of the KAT6B variant. The one woman affected with breast cancer found to be negative for the variant developed breast cancer in her 70s; she may represent a phenocopy. Interestingly, the exome sequencing identified an obligate gene carrier in generation II; an unaffected mother with two affected daughters who carry the variant. The KAT6B mutation remains under investigation. As part of a larger consortium, COMPLEXO, with Memorial Sloan-Kettering Cancer Center, Mayo Clinic, University of Pennsylvania, and other institutions, we at Creighton are initiating capture-resequencing of additional cases and matched controls to validate these findings. Additionally, we will better elucidate the clinical spectrum of KAT6B mutations to address the question: Are they "private" to a single family or more general?

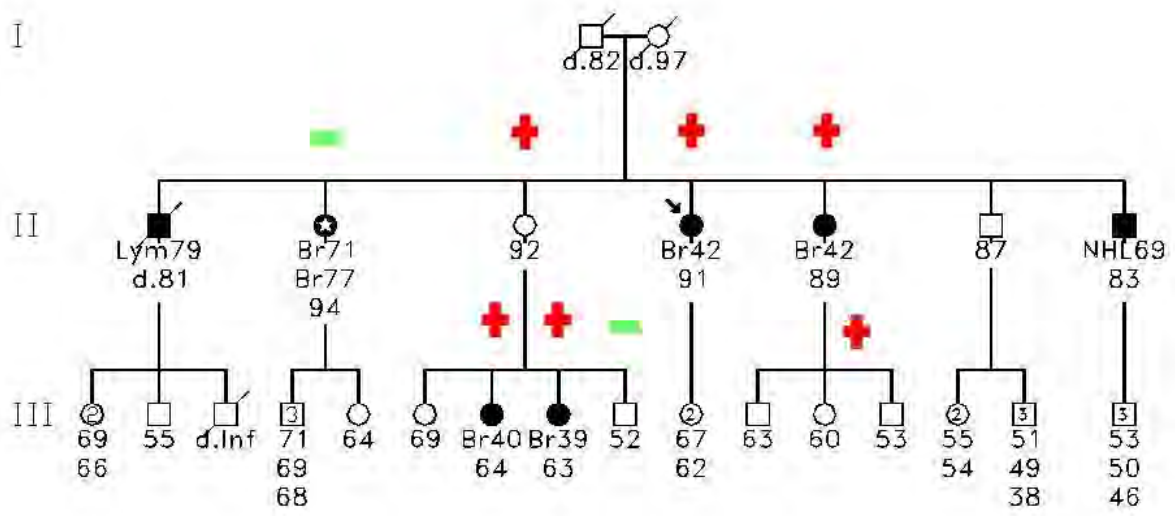

Figure 1. BRCAx FBC family with identified segregating KAT6B candidate variant

The Lynch program and Wang are collaborative participants in the international COMPLEXO consortium, which aims to identify unknown predispositions in BRCAx familial breast cancer.

During the past year, Wang and his team have analyzed genetic samples provided from our registered BRCAx breast cancer families. A recent publication[1] from this analysis on 22 probands from BRCAx families described the presence of family-specific, novel, deleterious germline variants in each family. Many were shared between breast cancer-affected family members but not found in affected women in unrelated families. These findings support that the predispositions for many BRCAx familial breast cancer families can be specific to each family and not shared among many BRCAx families. The application of a family-focused approach has the ability to detect new predisposition genetic mutations that may be unique to a given family.

An additional publication by Wang and his team[17] described the results of genetic studies in BRCAx and BRCA1+ families. One-hundred seven exome data sets were analyzed from women who were either part of a BRCA1+ or BRCAx family. Two novel heterozygous mutations were found on PALB2 (c.2014G?C, p.E672Q and c.2993G>A, p.G998E). Interestingly, both of these mutations were found in BRCA1+ and BRCAx families. They concluded that mutations in PALB2 can occur independent of the status of BRCA1 mutations; they highlight the importance to test for PALB2 mutations even when a BRCA1 is identified within a family.

C. Significance

About 10% of breast cancers are clustered in families (familial breast cancer, FBC), indicating the presence of genetic predisposition. BRCA1 and BRCA2 germline mutations, as well as all other germline mutation predispositions currently known, account for only 30-40% of FBC. Our BRCAx research is aimed at the discovery of unknown mutations that could help define the cancer risk of FBC families.

Figure 2 depicts a family that is representative of the 179 ascertained and cancer-verified families with stored samples to date. Families such as these are vital to the discovery of novel predisposition genetic variants.

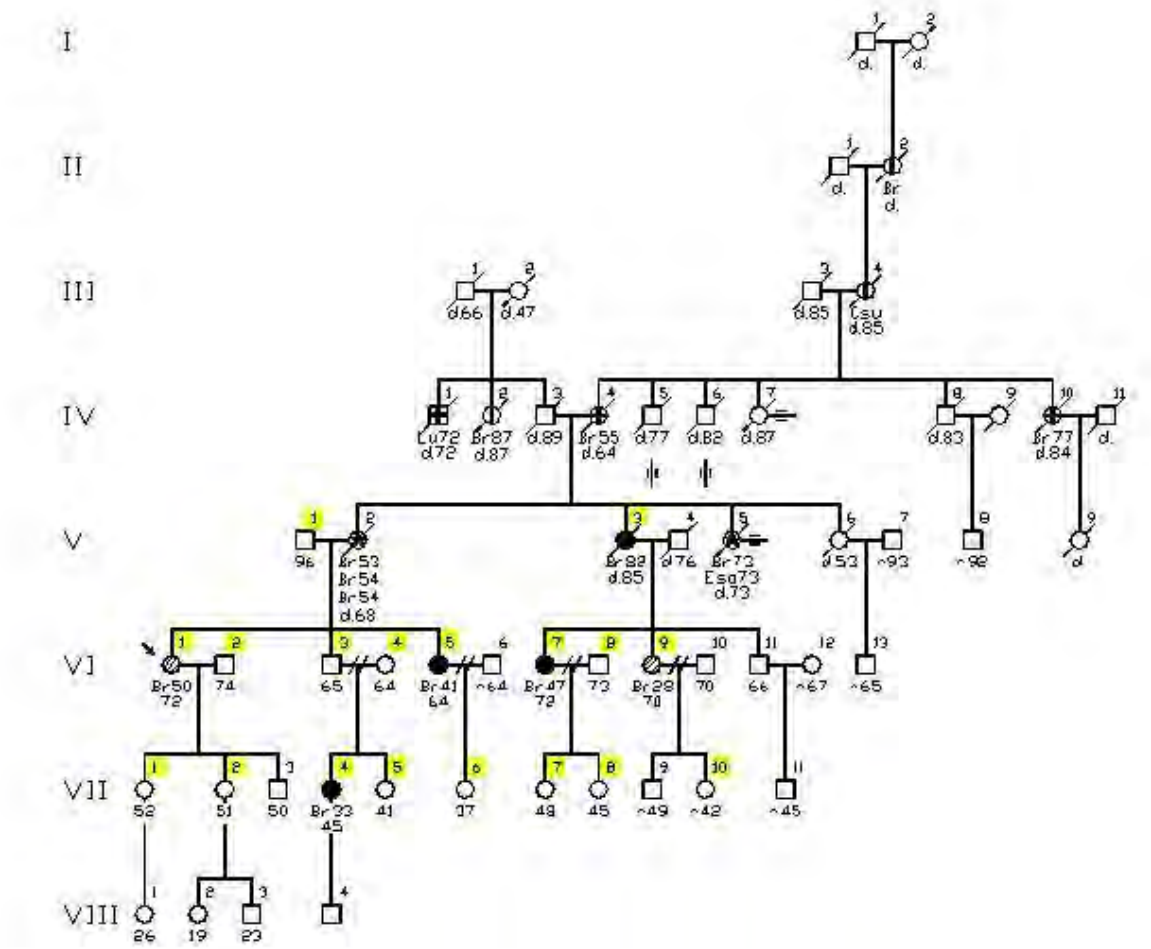

Figure 2: BRCAx FBC family targeted for exome sequencing. Samples are stored on highlighted family members.

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Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

MOLECULAR MECHANISMS AND NOVEL TARGETS IN CANCER RESEARCH PROGRAM PROGRESS REPORT

Laura Hansen, PhD, Director

Project 1: Novel Biomarkers and Therapeutic Targets for Nonmelanoma Skin Cancer

Principal Investigator: Laura Hansen, PhD

I. Progress Report Summary

A. Specific Aims

The aims have not been modified.

B. Studies and Results

Progress has been made on all three *specific aims* in the past year, as described below:

Aim 1. To determine how 14-3-3 heterodimerization, CDC25A binding, and cytoplasmic relocalization suppress SCC apoptosis. Both CDC25A and 14-3-3 family member over-expression in cancer can be associated with worse prognosis. We previously demonstrated an increase in CDC25A and 14-3-3 epsilon levels and a shift from nuclear CDC25A and 14-3-3 epsilon localization in malignant skin tumors from human clinical specimens and from UV-exposed mice when compared to normal skin. Cumulatively, our previous data revealed that increased cytoplasmic CDC25A in malignant skin cancers compared to normal skin keratinocytes suppressed apoptotic cell death. In the past year we performed a series of experiments to determine whether the anti-apoptotic effect of CDC25A was specific for skin cancer cells compared to normal skin keratinocytes. As shown in Fig. 1, forced expression of CDC25A reduced apoptosis in two different human skin cancer cell lines (SCC12B.2 and SCC13) but not in nontumorigenic human skin keratinocytes (HaCaT), while silencing of CDC25A increased apoptosis in the SCC cells but not in the nontumorigenic keratinocytes. Experiments using two different CDC25A inhibitors, Vitamin K3 and NSC663284, produced similar results, with increased toxicity to SCC cells (SCC12B.2) compared to nontumorigenic HaCaT cells (Figure 2).

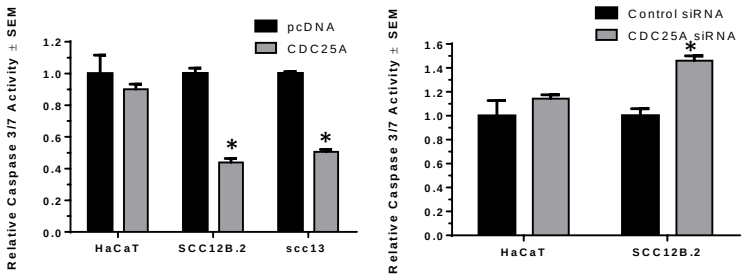


Figure 1. CDC25A suppresses apoptosis in SCC but not nontumorigenic human skin keratincoytes. The indicated cell lines were transfected with *CDC25A* (left) or with *CDC25A*-targeted siRNA (right) and apoptosis measured 24 h (left) or 48 h (right) later using a Caspase activity assay. N=3. Representative of ≥2 experiments performed.

We previously had shown that CDC25A binding to 14-3-3 is necessary for its anti-apoptotic effect, and also that silencing of 14-3-3 epsilon increased apoptotic cell death in SCC cells. Thus, we proposed investigation into 14-3-3 regulation of CDC25A function and localization. We hypothesized that 14-3-3 may relocalize CDC25A to the cytoplasm of skin cancers. CDC25A is known to bind to 14-3-3 epsilon, beta, and eta. These isoforms can heterodimerize with each other and with other family members. 14-3-3 epsilon, in particular, can heterodimerize with 14-3-3 β, γ, ζ, τ, or η (1). Thus, in the past year, we have focused on determining which 14-3-3 family members are expressed in skin and skin tumors, and in particular on which of them have an

increased cytoplasmic localization in skin tumors, which would allow for binding to cytoplasmic CDC25A.

To begin to investigate 14-3-3 function in skin and skin cancer, we first assessed 14-3-3 isoform expression and localization in both mouse and human skin cancers, as well as in human SCC and normal keratinocyte cell lines. As shown in Fig. 3 (and data not shown), all 14-3-3 family members except for 14-3-3 beta were detected in both mouse and human skin tumors using immunohistochemistry. 14-3-3 epsilon was relocalized to the cytoplasm in premalignant and malignant skin cancers from both a mouse model and humans (Fig. 3 top). 14-3-3 zeta exhibited a similar pattern of expression and localization in the analyses (Fig. 3 top). 14-3-3 eta and gamma were relocalized from primarily nuclear to mostly cytoplasmic localization in the malignant mouse and human tumors (Fig. 3 top). 14-3-3 theta, in contrast, was decreased in mouse and human tumors when compared to skin. 14-3-3 beta was not detected in our immunohistochemistry experiments, in either mouse or human skin or tumors, with either of two commercially available antibodies (not shown). However, 14-3-3 beta was easily detected using immunoblotting of nontumorigenic keratinocytes or human SCC cell lines (Fig. 3 bottom). 14-3-3 beta was primarily in the nucleus of human SCC lines (Fig. 3 bottom). Consistent with the immunostaining, 14-3-3 epsilon levels were generally increased in the SCC cells compared to the normal keratinocytes and shifted to a more cytosolic localization in the SCC cells as well (Fig. 3 bottom). 14-3-3 eta, gamma, theta, and zeta were increased in the SCC lines compared to normal keratinocytes and were primarily present in the cytosolic fraction (Fig. 3 bottom). Thus, 14-3-3 eta, gamma, and zeta are most likely to heterodimerize with 14-3-3 epsilon in the cytosolic compartment of skin malignancies, while 14-3-3 beta may form heterodimers in the nucleus. Since 14-3-3 sigma only forms homodimers, we have not included our results with it here.

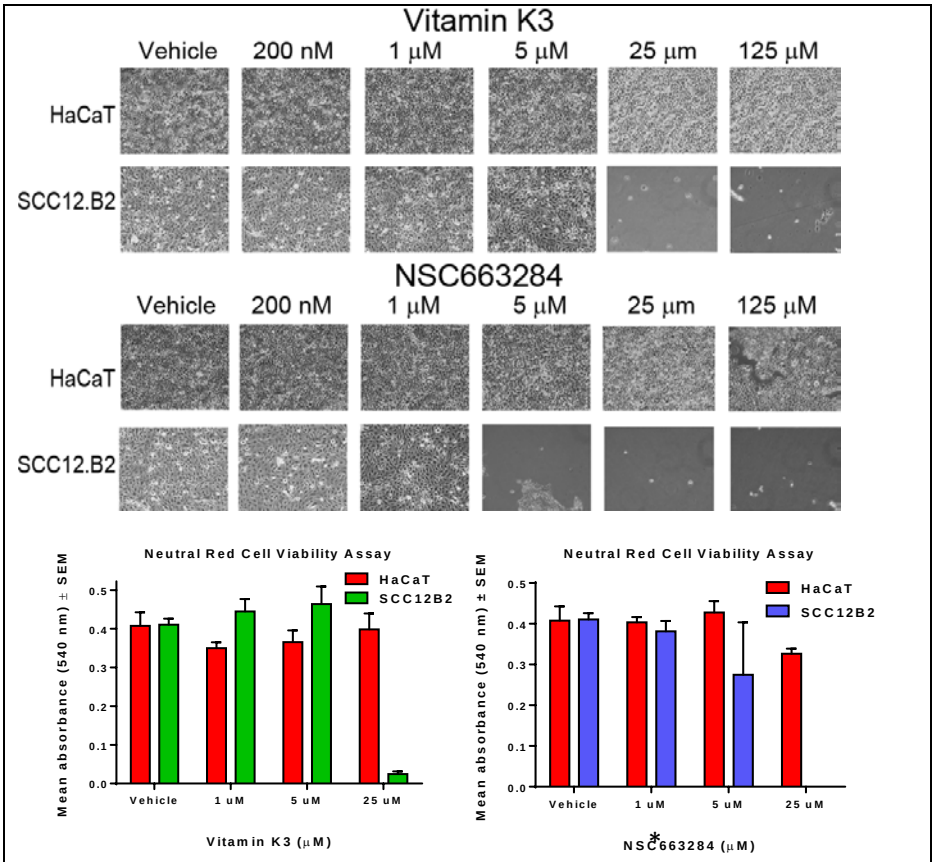


Figure 2. Nontumorigenic skin keratinocytes are less sensitive than SCC cells to CDC25A inhibition. Cells were treated with the indicated CDC25A inhibitor at the indicated concentrations for 24 h. Phase contrast images (top) of representative wells and neutral red cell survival assay results (bottom) are shown. N=3 wells/treatment. Experiments are representative of several performed.

In the past year, we have also developed protocols for the specific silencing of 14-3-3 isoforms in our cell culture models, as shown in Fig. 4A, in order to begin to determine which 14-3-3 interactions result in CDC25A localization to the cytosol. Silencing of 14-3-3 epsilon, zeta, and eta resulted in a modest increase in nuclear CDC25A (Fig. 4B-D). Experiments in the coming

year will silence 14-3-3 members in combination to determine whether a larger effect on CDC25A localization can be generated, or whether 14-3-3 binding has other effects on CDC25A function.

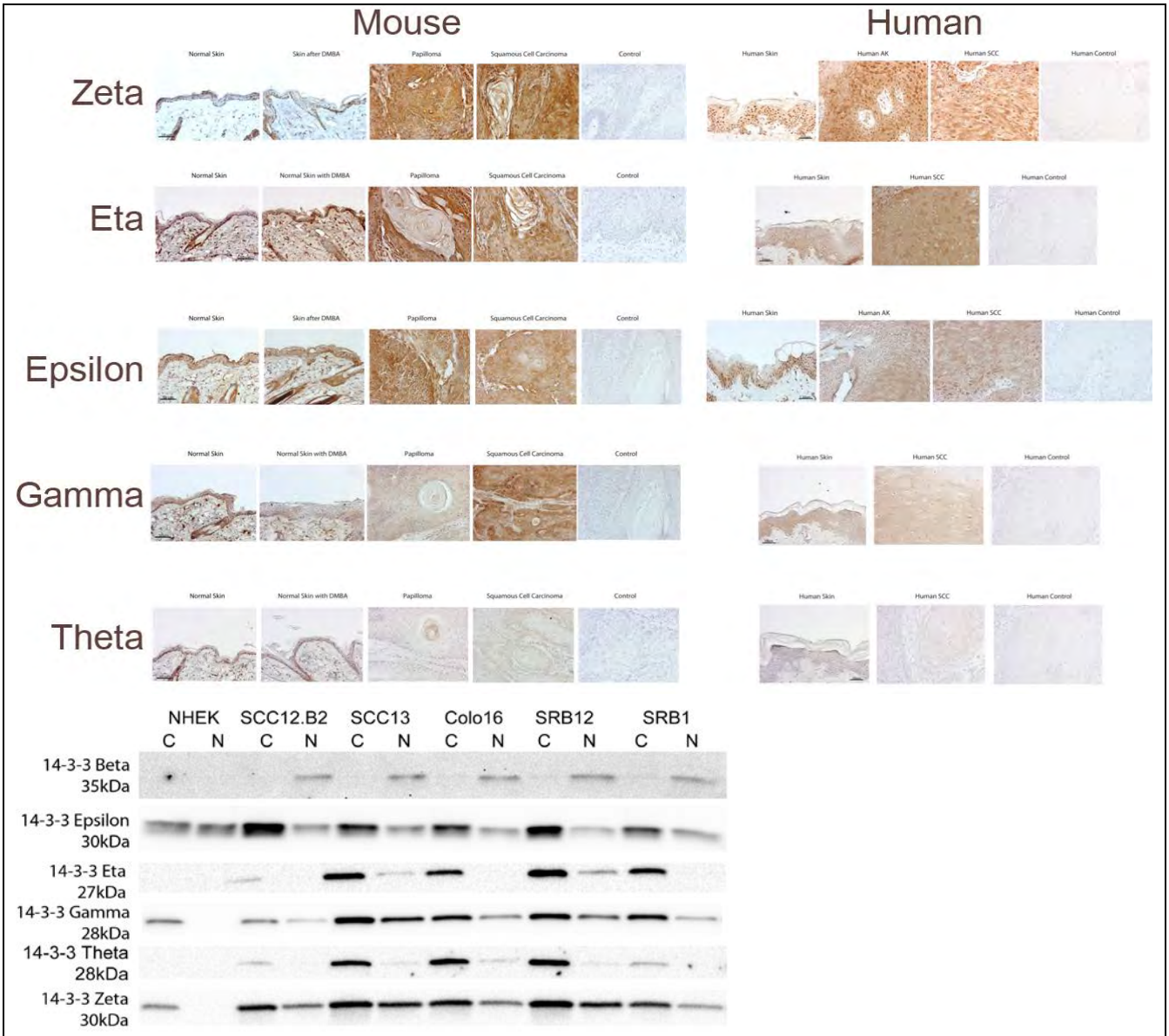


Figure 3. 14-3-3 expression and localization in human and mouse nonmelanoma skin tumors and cell lines. Top) Immunohistochemistry for 14-3-3 isoforms (brown) with hematoxylin counterstain (blue) in mouse (left) and human (right) skin, premalignant papillomas (mouse) and actinic keratoses (human), and SCC. Image on right-hand for both species is negative immunoglobulin control. Bottom) Immunoblotting for 14-3-3 isoforms in NHEK (normal human epidermal keratinocytes) and human skin SCC cell lines after fractionation of cytosolic (C) and nuclear (N) proteins. DMBA=7,12-dimethylbenz[a]anthracene.

Aim 2. To develop a genetic model for targeting 14-3-3ε interactions against skin carcinogenesis. In this aim, a mouse model with skin-targeted deletion of the gene for 14-3-3ε will be developed and the effect on skin cancer tested using the well-characterized model of chemical carcinogen treatment of mouse skin. Serendipitously, Toyo-oka et al. (2) reported the development of a conditional *Ywhae* mouse in the fall of 2014. As described in Fig. 5A-C, the resulting the resulting *Ywhae*$^{fl/fl}$ mouse has loxP sites flanking exons 3-4 of the *Ywhae* gene. Cre recombinase expression via breeding with an *hGFAP-Cre recombinase* mouse results in targeted deletion of *Ywhae* in the developing cortex (Fig. 5D-E). We are in the process of importing the *Ywhae*$^{fl/fl}$ mice to the Creighton University Animal Resource Facility for use in the

experiments proposed in Aim 2. The animal facility anticipates one of the quarantine microisolators will be available for these mice around the end of September, at which time the mice will be shipped here. Once on campus, the *Ywhae*$^{fl/fl}$ mice will be crossed with *Krt14-Cre recombinase* mice that we have already established in the facility for this work, to direct Cre recombinase expression to the keratinocytes of the epidermis and provide for skin-targeted deletion of *Ywhae*.

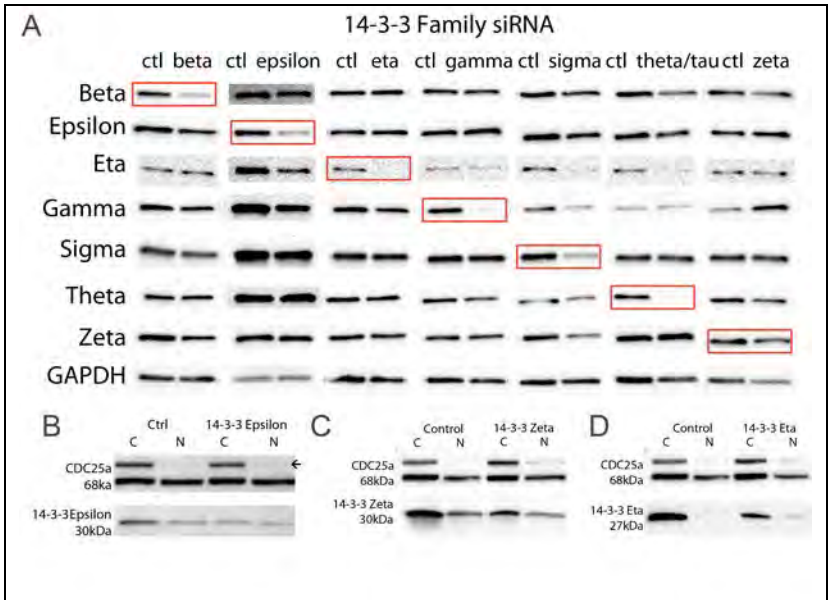


Figure 4. Silencing of 14-3-3 isoforms slightly increased nuclear CDC25A in human SCC cell line SCC12B.2. A) 14-3-3 isoforms indicated at top or control siRNA (ctl) were transfected into SCC12.B2 cells and immunoblotting for the indicated proteins (labels on left-hand column) performed using total protein extract 2 d later. B-D) The indicated 14-3-3 isoforms were silenced in SCC12.B2 cells and immunoblotting for CDC25A and 14-3-3 isoforms performed using nuclear (N) and cytosolic (C) fractions 2 d later.

Aim 3. To develop novel agents for targeting of 14-3-3 epsilon for use in skin cancer treatment and prevention. In this aim, *de novo* peptides targeting 14-3-3 epsilon heterodimerization will be developed using *in silico* analyses. The peptides with the highest binding affinities from *in silico* screening will be tested *ex vivo* to identify and validate inhibitors of CDC25A and 14-3-3ε interactions and the impact on SCC cell death.

The most stable heterodimers formed by 14-3-3 epsilon protein are with the eta, gamma, theta/tau, and zeta isoforms (3). Since no X-ray structure is available for any of these heterodimers, initially the dimeric structures were built from the individual monomeric X-ray structures using YASARA. Their structural stability was studied by molecular dynamics (MD)

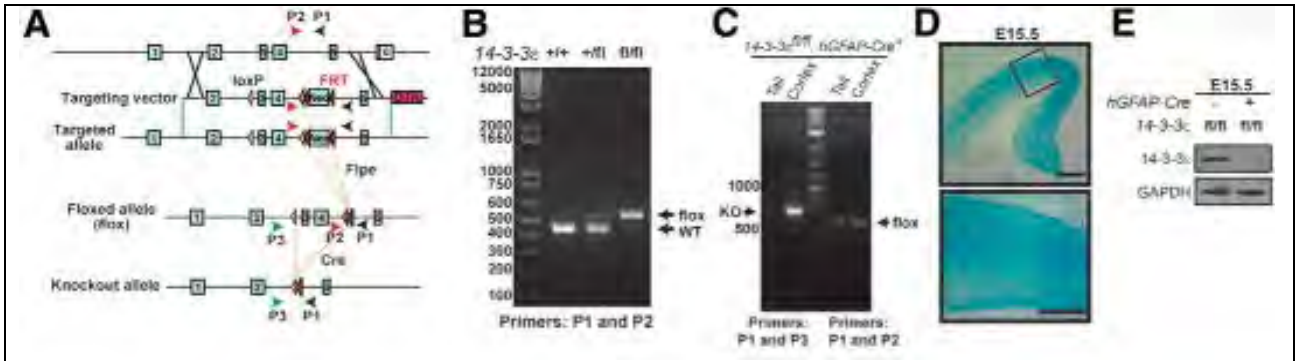


Figure 5. Targeting of *Ywhae* (14-3-3 epsilon) in the mouse. A) Schematic illustration of the gene targeting of the *Ywhae* gene**.** Numbers indicate exons. Red and yellow arrowheads indicate FRT and loxP sites, respectively. P1, 2, and 3 indicate primers. B) PCR genotyping of conditional *Ywhae* knockout mice. C) PCR genotyping of *Ywhae*$^{fl/fl}$*/hGFAP-Cre*$^{+}$ mice at P15. D) Cre recombinase expression in the cortex of E15.5 mice. E) Immunoblotting for 14-3-3 epsilon in E15.5 embryonic cortex. Adapted from Toyo-oka et al. (2).

simulations using the GROMACS 5.0.5 and the CHARMM36 force field. Initially, we planned to use the AMBER ff99SB-ILDN-NMR force field parameters, but the former describes better structural properties of phosphopeptides and their interactions with proteins that will be studied at later stage of the project. For each heterodimer, 250 ns MD simulations were performed and the

trajectories were analyzed by using cluster analysis, dynamic cross correlation, root mean square fluctuations (RMSF), and conformational entropy calculations.

The epsilon-eta and epsilon-theta/tau dimers of 14-3-3 are reportedly equally abundant in the cytosol, whereas the epsilon-gamma and epsilon-zeta dimers mostly occur in the nucleus. Therefore, we selected the epsilon-theta/tau and epsilon-zeta dimers for molecular docking simulations. There are two possible specific docking sites on the dimers (Figure 6), which we called site 1 and 2 (S1 and S2).

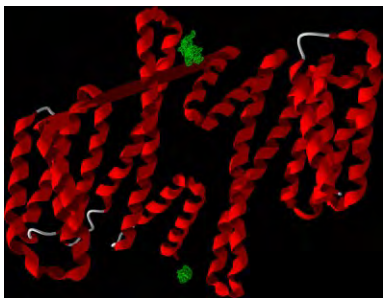


Figure 6. Docking sites on the 14-3-3 heterodimers. Proteins are in red ribbon representation; left is the epsilon and right protein is either the theta or zeta isoform. The docking sites are marked with green meshed surfaces. Site 1 (S1) on top and site 2 (S2) on the bottom.

For the docking simulations, the representative dimer structure of the most populated cluster of the 250 ns trajectory was used. We developed a virtual tetrapeptide library, containing all possible combinations of any standard amino acid residues (19^4), with 130,321 different structures. The Cys residue was not included to prevent synthetic problems that might occur at a latter stage of the project. Peptides had either free termini or were N- and C-terminally Ac- and -NH_2 protected, respectively. These peptides were docked on the sites of the heterodimers using the Molegro Virtual Docker software. Tetrapeptides docked favorably to the epsilon-theta/tau dimer S1 site 41 and to the S2 site 24. To the epsilon-zeta dimer S1 site 27, tetrapeptides docked favorably; the S2 site of the epsilon-zeta dimer turned out to be too narrow and no successful docking was achieved. On the basis of the Molegro scoring function and pose geometry, we selected three peptides each for the S1 and S2 sites of the epsilon—theta/tau dimer and two peptides for the S1 site of the epsilon-zeta dimer (Table 1). The stability of the eight selected peptide 14-3-3 complexes were studied by 250 ns MD simulations. Out of the eight complexes, seven peptides dissociated from the initial site and either did not form new stable complex or the peptide docked to the classical peptide binding groove of the 14-3-3epsilon protein. The Trp-Tyr-Trp-Lys-NH_2 tetrapeptide did not dissociate from the S1 site of the epsilon-zeta dimer using the initial MD simulation. Therefore, further investigation of the stability of this complex is underway.

A new series of docking simulations in which the peptide library is docked to S1 and S2 only of the 14-3-3e monomer are in progress.

Table 1. Selected peptides and their docking energies.

	ε–τ dimer		ε–ζ dimer	
	Peptide	E_{rel}/Kcal mol^{-1}	Peptide	E_{rel}/Kcal mol^{-1}
	Ac-His-Asn-Asp-Trp-NH_2	-109.633	Ac-Trp-His-His-Ala	-116.327
S1	Thr-Thr-Asp-His	-109.286	Trp-Tyr-Trp-Lys-NH_2	-115.819
	His-Ser-Arg-Asp	-108.898		
	Gln-Trp-Tyr-Glu	-106.682		
S2	Thr-Arg-Asp-Ser-NH_2	-106.456		
	AcPhe-Gln-Lys-His-NH_2	-105.187		

C. Significance

The proposed project is expected to provide substantial new information about 14-3-3 signaling,

interactions with CDC25A, and functions in skin cancer, provide proof-of-principle evidence of the efficacy of targeting 14-3-3 and CDC25A functions and interactions for skin cancer treatment and prevention, and further the development of novel agents useful for the treatment of skin and other cancers. The proposal overall is designed to provide critical preliminary data and reagents necessary for submission of a successful R01 application investigating 14-3-3 and CDC25A as targets for skin cancer treatment and/or prevention.

II. List of refereed publications germane to this project from 7/1/2014–6/30/2015

1. Hammiller, B.O., El-Abaseri, T.B., Dlugosz, A.A., and **Hansen, L.A.** A method for the immortalization of newborn mouse skin keratinocytes. *Frontiers in Oncology,* 2015. doi: 10.3389/fonc.2015.00177

2. Shirley, S.H., Hammiller, B., **Hansen, L.A.**, Crysup, B., Hudson, L.G., and Kusewitt, D.F. Role of the epidermal growth factor receptor in ultraviolet radiation induction of Snail family transcription factors. *J Dermatol Sci*, 76(2):149-51, 2014. doi: 10.1016/j.jdermsci.2014.09.001

3. Bichsel, K.J., Hammiller, B., Trempus, C.S., Li, Y., **Hansen, L.A.** The epidermal growth factor receptor decreases Stathmin 1 levels to trigger catagen entry in the mouse. Submitted, 2015.

III. List of extramural grants submitted from 7/1/2014–6/30/2015

INBRE PI: M. Nichols (CoI: Hansen)
Dates: 5/1/15-4/30/16
Title: Metabolic imaging of disease progression in skin cancer by FLIM
Award: $92,300 (total direct)

CURAS Project PI: Hansen
Title: Cytoplasmic CDC25A localization and suppression of apoptosis in cancer cells.
Dates: 7/1/15-6/30/16
Award: $5,000

ProTransit Nanotherapy Project PI: Hansen
Title: The Protective Efficacy of PLGA Nanoparticles Encapsulating Antioxidant Enzymes against UV Radiation Exposure
Dates: 7/1/14-12/30/15
Award: $5868

LB506 PI: Hansen
Title: Estrogen receptor alpha and squamous cell carcinoma of the skin
Proposed Dates: 7/1/2015-6/30/2016
Proposed Award: $50,000

NIH R01 PI: Hansen
Title: Mechanisms of UV-induced skin carcinogenesis (Competitive Renewal)
Proposed Dates: 2015-2020
Proposed Award: $1,838,406

NIH STTR PI: Madsen (Multi-PI Hansen)
Title: Developing Pro-NP for skin cancer prevention
Proposed Dates: 9/1/15-8/31/16
Proposed Award: CU subcontract $97,523

IV. List of extramural grants awarded from 7/1/2014–6/30/2015

CURAS Project PI: Hansen
Title: Cytoplasmic CDC25A localization and suppression of apoptosis in cancer cells.
Dates: 7/1/15-6/30/16
Award: $5,000

ProTransit Nanotherapy Project PI: Hansen
Title: The Protective Efficacy of PLGA Nanoparticles Encapsulating Antioxidant Enzymes against UV Radiation Exposure
Dates: 7/1/14-12/30/15
Award: $5868

INBRE PI: M. Nichols (CoI: Hansen)
Dates: 5/1/15-4/30/16
Title: Metabolic imaging of disease progression in skin cancer by FLIM
Award: $92,300 (total direct)

V. References

1. Chaudhri M, Scarabel M, Aitken A. Mammalian and yeast 14-3-3 isoforms form distinct patterns of dimers in vivo. Biochem Biophys Res Commun 2003; 300(3): 679-85

2. Toyo-oka K, Wachi T, Hunt RF, et al. 14-3-3epsilon and zeta regulate neurogenesis and differentiation of neuronal progenitor cells in the developing brain. J Neurosci 2014; 34(36): 12168-81

3. Yang X, Lee WH, Sobott F, et al. Structural basis for protein-protein interactions in the 14-3-3 protein family. Proc Natl Acad Sci U S A 2006; 103(46): 17237-42

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

MOLECULAR MECHANISMS AND NOVEL TARGETS IN CANCER RESEARCH PROGRAM PROGRESS REPORT
Laura Hansen, PhD, Director

Project 2: LincRNAs and Oncogenic Activation of NF-κB in Prostate Cancer Progression
Principal Investigator: Xian-Ming Chen, MD, MSc

I. Progress Report Summary

A. Specific Aims

Prostate cancer (PCa) is one of the most frequently diagnosed malignancies in men in the United States, resulting in about 200,000 new cases and 30,000 deaths each year. Androgen deprivation therapy remains the most prevalent treatment, but unfortunately this therapy will fail for most patients and the tumor will progress to an advanced stage, commonly referred to as androgen refractory or castration-resistant prostate cancer (CRPC). Underlying mechanisms for this progression for PCa are unclear and there is currently no curative treatment.

The long intergenic ncRNAs (lincRNAs), non-coding transcripts (> 200 nt) from the intergenic regions of annotated protein-coding genes, play a key regulatory role across diverse biological functions. Recent studies have demonstrated that a subset of lincRNAs, so-called prostate cancer-associated ncRNA transcripts (PCATs), may play an active role in prostate cancer progression. The overriding hypothesis of this research project is that oncogenic activation of NF-κB signaling downregulates expression of metastatic suppression genes to promote CRPC progression of PCa through IKKα/lincRNA/PRC2-mediated epigenetic chromatin remodeling, a process facilitated by the nuclear-to-cytoplasmic translocation of 14-3-3epsilon and 14-3-3eta (**Figure 1**). We aim to investigate the following hypotheses: (1) nuclear accumulation of IKKα and nuclear-to-cytoplasmic translocation of 14-3-3 proteins facilitate assembly of lincRNAs to the PRC2 complex, promoting PRC2-mediated epigenetic chromatin remodeling in PCa cells (Aim 1); (2) designed RNA aptamers can disrupt specific lincRNA-EZH2 interactions to interfere with PRC2-mediated epigenetic chromatin remodeling (Aim 2); and (3) androgen deprivation induces nuclear accumulation of IKKα and nuclear-to-cytoplasmic translocation of 14-3-3 proteins, promoting PCa CRPC progression through modulation of lincRNA/PRC2-mediated epigenetic chromatin remodeling (Aim 3). The hypotheses and Specific Aims have not changed. During the 2014-2015 budget year, as detailed below, we have begun to address all the hypotheses as proposed in our Specific Aims using an array of biochemical and molecular biological approaches, including qRT-PCR and Northern blot analysis of lincRNA expression, gene silencing with siRNAs, and ChIP/RIP assay for lincRNA-protein interactions.

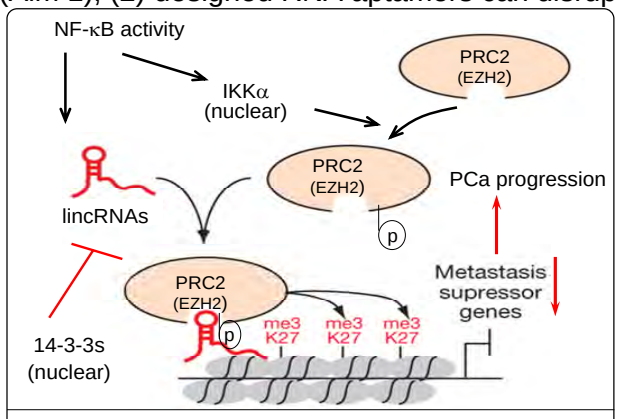


Figure 1. Hypotheses and novel concepts. Oncogenic activation of NF-κB signaling downregulates expression of metastatic suppression genes to promote CRPC progression of PCa through IKKα/lincRNA/PRC2-mediated epigenetic chromatin remodeling, a process facilitated by the nuclear-to-cytoplasmic translocation of 14-3-3 transcription factors.

B. Studies and Results

We have performed three groups of experiments to address our central hypothesis and Specific Aims. The first group of experiments has been focused on the nuclear accumulation of IKKα

and nuclear-to-cytoplasmic translocation of 14-3-3 proteins associated with lincRNA assembly to the PRC2 complex in PCa cells induced by NF-kB activation (i.e., stimulation by lymphotoxin B, related to Specific Aim 1). We have utilized several PCa cell lines (i.e., LNCaP and C4-2B cells) for our initial *in vitro* experiments. Our resulting data indicate the accumulation of activated IKKα in nuclei and a nuclear-to-cytoplasmic translocation of 14-3-3 proteins, as well as altered expression of lincRNAs, in cells after stimulation by lymphotoxin B (**Figure 2**). During the next budget year, we will determine the effects of nuclear accumulation of IKKα and nuclear-to-cytoplasmic translocation of 14-3-3 proteins on lincRNA assembly to the PRC2 complex and, subsequently, PRC2-mediated epigenetic chromatin remodeling. We will use an IKKa mutant to determine whether nuclear accumulation of IKKα is required for lincRNA assembly. LincRNA candidates have already been identified in our second group of experiments detailed below. We will also clarify whether nuclear-to-cytoplasmic translocation of 14-3-3 proteins is involved in the assembly of lincRNAs to the PRC2 complex.

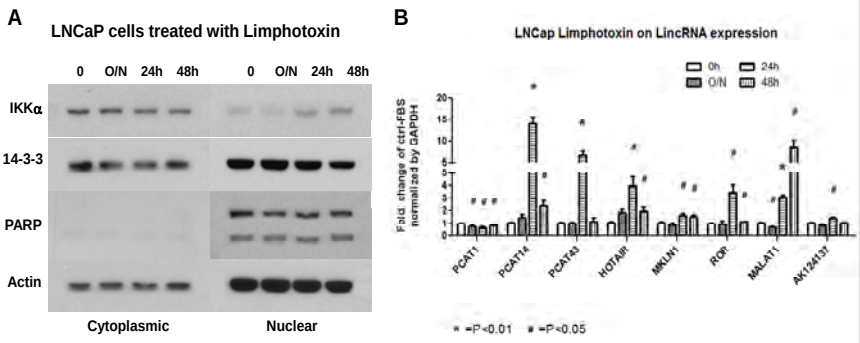


Figure 2. (A) Accumulation of activated IKKα in nuclei and a nuclear-to-cytoplasmic translocation of 14-3-3 proteins in LNCaP cells after stimulation by lymphotoxin B, as assessed by Western blot. **(B).** Altered expression of selected lincRNAs was detected in cells after lymphotoxin B stimulation as by real-time PCR.

Our second group of experiments was performed to identify those lincRNAs that are specifically assembled into the PRC2/EZH2 complex in PCa cells upon NF-kB activation by lymphotoxin B (related to Specific Aim 2). During the last budget year, we identified a panel of lincRNAs that were upregulated and assembled into the PRC2 complex in PCa cells upon lymphotoxin B stimulation using glutaraldehyde crosslinking RNA-binding immunoprecipitation (RIP) assay (**Figure 3**). During the next budget year, we will determine the regions of selected lincRNAs and EZH2 required for their interactions. We will generate a series of deletion mutants of lincRNA candidates and EZH2. Plasmids encoding HA-tagged full-length (pcDNA-EZH2, Addgene) or deletion mutants of EZH2 will be co-transfected with plasmids encoding the full-length or deletion mutants of lincRNA candidates. Results of these experiments will provide the basis for the design of RNA aptamers to disrupt specific lincRNA-EZH2 interactions to interfere with PRC2-mediated epigenetic chromatin remodeling in PCa cells.

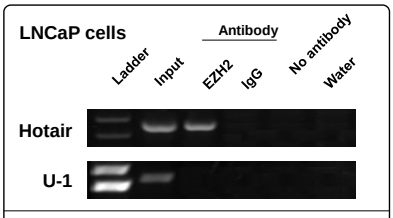


Figure 3. RIP analysis of induced assembly of lincRNAs into the PRC2 complex in LNCaP cells. Representative gel shows the presence of a lincRNA Hotair, but not a control RNA U-1, in the immunoprecipates with anti-EZH2.

In the third group of experiments, we began to explore the molecular mechanism underlying PCa progression after androgen deprivation through modulation of lincRNA/PRC2-mediated epigenetic chromatin remodeling (related to Specific Aim 3). During the last budget year, we have fully characterized the intracellular location of IKKα and 14-3-3 proteins and assembly of lincRNAs to the PRC2 complex in PCa cells after androgen deprivation (**Figure 4A-C**). We have successfully established the Chromatin Isolation by RNA Purification (ChIRP) approach to measure the recruitment of lincRNAs to specific gene loci of interest (**Figure 4D**). During the next budget year, we will determine the enrichment of specific lincRNAs and PRC2 complex to these metastatic suppressive gene loci in PCa cells (under androgen-deprived condition) stably expressing: i) the IKKα-NLS mutant; ii) the EZH2-deficient mutant; iii) shRNAs to each lincRNA; and iv) designed RNA aptamers to each lincRNA. We anticipate that aberrant translocation of

IKKα and14-3-3 proteins is associated with lincRNA/PRC2-mediated transrepression of metastatic suppressive genes in PCa cells after androgen deprivation.

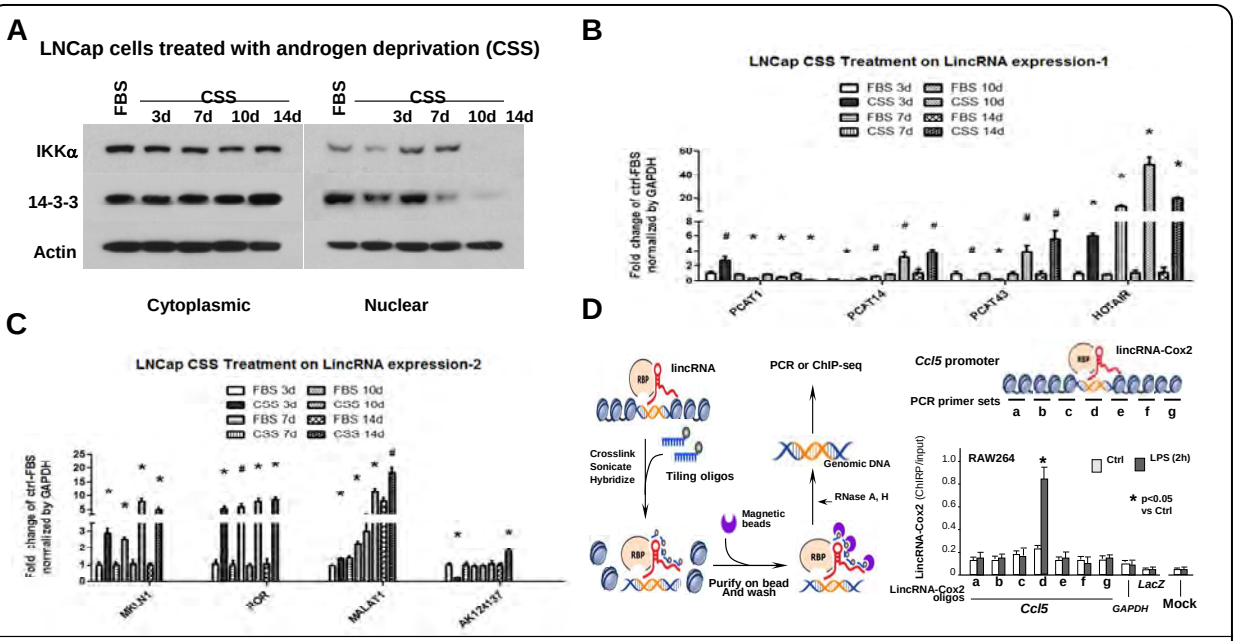


Figure 4. (A) Accumulation of activated IKKα in nuclei and a nuclear-to-cytoplasmic translocation of 14-3-3 proteins in LNCaP cells after androgen deprivation, as assessed by Western blot. **(B**) and **(C).** Altered expression of selected lincRNAs was detected in cells after androgen deprivation by real-time PCR. (D). Workflow and representative data of ChIRP analysis. Chromatin is crosslinked to lincRNA:protein adducts. A pool of biotinylated tiling probes are hybridized to target lincRNA, and chromatin complexes are purified using magnetic streptavidin beads. The resultant genomic DNA fragment(s) are validated by real-time PCR.

In addition, in parallel experiments, we have measured the nuclear accumulation of IKKα and nuclear-to-cytoplasmic translocation of 14-3-3 proteins associated with lincRNA assembly to the PRC2 complex during tumorsphere culture of PCa cells. Our results indicate a different pattern of lincRNA assembly to the PRC complex in PCa cells during tumorsphere formation. We speculate that lincRNAs may also play a role in the cancer stem-like transformation during PCa progression.

C. Significance

Our proposal aims to investigate the hypothesis that lincRNAs link oncogenic NF-kB activation and cytoplasmic 14-3-3 accumulation with PRC2-mediated epigenetic chromatin remodeling for metastatic progression of PCa. Several novel concepts will emanate from the proposed experiments addressing this central hypothesis. First, results from our proposed experiments will provide new mechanistic insights into how aberrant translocation of IKKα and 14-3-3 proteins promotes PCa progression. We will determine how aberrant translocation of IKKα and 14-3-3 proteins influences lincRNA/PRC2-mediated chromatin remodeling. Second, we will design and evaluate the therapeutic potential of RNA aptamers to interfere with lincRNA-EZH2 interactions. Several RNA-binding proteins, including EZH2, have been implicated in the pathogenesis of tumorigenesis, but the underlying mechanisms are unclear. We will test the interactions between lincRNAs and EZH2 in gene transcription in PCa progression and investigate the potential functional interference using designed RNA aptamers. Understanding how RNA-binding proteins are involved in the associated gene transcription and how it may be interfered with by RNA aptamers will provide new insights into therapeutic development.

II. Publications germane to this project from July 1, 2014–present.

1. Hu G, Gong AY, Wang Y, Ma S, Chen X, Chen J, Su C, Shibata A, Strauss-Soukup JK, Kristen M. Drescher KM, and Chen X-M. LincRNA-Cox2 promotes late inflammatory gene transcription through modulating SWI/SNF-mediated chromatin remodeling. (Under review by *Cell Reports*)

2. Tong Q, Gong AY, Lin C, Ma S, Chen J, Hu G, Strauss-Soukup JK, and Chen X-M. LincRNA-Cox2 Modulates TNFα-induced transcription of *Il12b* gene in epithelial cells through regulation of Mi-2/NuRD-mediated epigenetic histone modifications. (Under review by *FASEB J*)

III. List of extramural grants submitted from 7/1/2014 – 6/30/2015

NIH/NIAID R01 AI116323
Title: Molecular Basis of Intestinal Cryptosporidiosis
PI: Xian-Ming Chen
Submitted on 09/01/2014

IV. List of extramural grants awarded from 7/1/2014 – 6/30/2015

Department of Defense - PC121561 Hypothesis Development Award
Title: LincRNAs and AR Reactivation after Androgen-Deprivation in Prostate Cancer Cells
PI: Xian-Ming Chen
Co-Invest, Yaping Tu
Funded (10/1/2013-9/29/2014)

LB595
Title: LincRNAs and Oncogenic Activation of NF-κB in Prostate Cancer Progression
PI: Xian-Ming Chen
Funded (07/01/14-06/30/15)

NIH/NIAID U01 AI095532-04
Title: Epithelial Exosomes and TLR-Mediated Mucosal Epithelial Defense
PI: Xian-Ming Chen
Funded (07/01/14-06/30/15)

NIH/NIAID R01 AI116323-01
Title: Molecular Basis of Intestinal Cryptosporidiosis
PI: Xian-Ming Chen
Funded (02/04/15-01/31/20)

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

MOLECULAR MECHANISMS AND NOVEL TARGETS IN CANCER RESEARCH PROGRAM PROGRESS REPORT
Laura Hansen, PhD, Director

Project 3: Novel Biomarkers and Potential Therapeutic Targets for Chronic Lymphocytic Leukemia (CLL)
Principal Investigator: Patrick Swanson, PhD

I. Progress Report Summary

A. Specific Aims

The original specific aims are as follows:

1. Determine S100A6 expression patterns and mechanism of action in murine and human CLL.
2. Establish a role for Hsp70-1 in promoting leukemic cell survival.
3. Investigate the role of 14-3-3ε and Cdc25A in CLL survival and progression.

One additional aim was added to complete studies started in the previous grant cycle and address reviewer comments to an NIH grant submitted in October 2014:

4. Determine the requirement of CD1d, IL10, and IL21-receptor expression on the development and B10-like function of $CD5^+$ B cells and hypogammaglobulinemia in dnRAG1 mice.

B. Studies and Results

Specific Aim 1:

This aim involved a collaboration with Drs. Tarantolo and Hauke to obtain primary human CLL samples for purification of leukemic cells to test whether S100A6 (calcyclin, a small Ca^{2+}-binding protein) is upregulated in human CLL. To date, we have consented, and obtained peripheral blood from, 18 CLL patient and 10 healthy controls, isolated PBMCs by double-density centrifugation, and enriched B cells (normal or CLL) by negative selection using MACS separation. Immunoblotting revealed that S100A6 expression is upregulated in human CLL (~2 fold; $p = 0.03$) from a spectrum of patients (Fig. 1A-B). This outcome is consistent with the ~2-fold upregulation of S100A6 expression in human CLL at the transcriptional level and the overexpression of S100A6 detected in $CD5^+$ B cells from CLL-prone mice. S100A6 is known to form a stable complex with Annexin A2 in normal murine $CD5^+$ B1 B cells, and was found to be upregulated in murine $CD5^+$ B cells from CLL-prone mice. However, in contrast to murine CLL, Annexin A2 was not significantly upregulated in human CLL (Fig. 1A-B). Preliminary experiments to detect Annexin A2 association with S100A6 in human CLL by co-immunoprecipitation have provided no evidence to support this interaction. Efforts to rule in or rule out association with other known S100A6 interaction partners are ongoing.

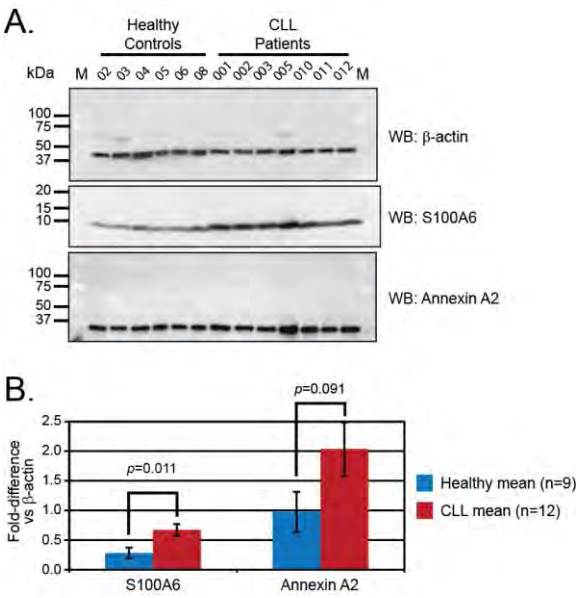


Figure 1. S100A6 but not Annexin A2 is overexpressed in human CLL. (A) RIPA lysates were prepared from MACS-isolated B cells obtained from healthy controls or CLL patients (10^5 cell equivalents) and analyzed by Western blotting (WB) to detect β-actin, S100A6, or Annexin A2. (B) Fold-change of S100A6 and Annexin A2 levels between CLL patients and healthy controls normalized to β-actin loading control.

Specific Aim 2:

We obtained several cell-permeable peptide inhibitors designed by collaborator Dr. Sandor Lovas to block the ATPase activity of Hsp70-1, and tested their ability to impair the growth of the myeloid leukemia line K562 *in vitro*. However, these inhibitors showed little effect on cell growth compared to

the positive control 2-phenylethynesulfonamide. Some concern about peptide degradation was raised which necessitates repeating the experiment using freshly prepared peptides.

Specific Aim 3:

Some preliminary immunohistochemical staining of spleen sections from 36 week-old wild-type, dnRAG1, Eμ-TCL1, and DTG mice has been performed using antibodies to 14-3-3 proteins, but the results have not yet been fully analyzed.

Specific Aim 4:

The majority of the efforts in the past year have been focused on completing analysis of 12- and 36-week-old wild-type, dnRAG1, Eμ-TCL1, and DTG mice that are CD1d-proficient or CD1d-deficient, and thus lack CD1d expression and CD1d-restricted natural killer T (NKT) cells. These studies were in progress at the end of the last program grant period. We have now completed these studies. In three different transgenic mouse models of benign or leukemic $CD5^+$ B cell expansion, we show that CD1d is differentially expressed on $CD5^+$ B cells between the three models, but loss of CD1d expression has no significant effect on $CD5^+$ B cell abundance or inducible IL10 expression in any of the models (Fig. 2, and data not shown). Interestingly, loss of CD1d expression reduced spontaneous IgG (but not IgM) production in the CLL-prone Eμ-TCL1 model (Fig. 3), and skewed splenic $CD4^+$ and $CD8^+$ T cell populations in the dnRAG1xEμ-TCL1 (DTG) model of accelerated CLL (Fig. 4). Unexpectedly, before leukemia onset, all three transgenic CD1d-deficient mouse strains had fewer splenic transitional B cells than their CD1d-proficient counterparts (Fig. 5), revealing a novel supporting role for NKT cells in B cell development in mice prone to $CD5^+$ B cell expansion. The results of this study have been described in a manuscript submitted for publication.

In response to reviewer critiques to an NIH grant submitted in October 2014, we have

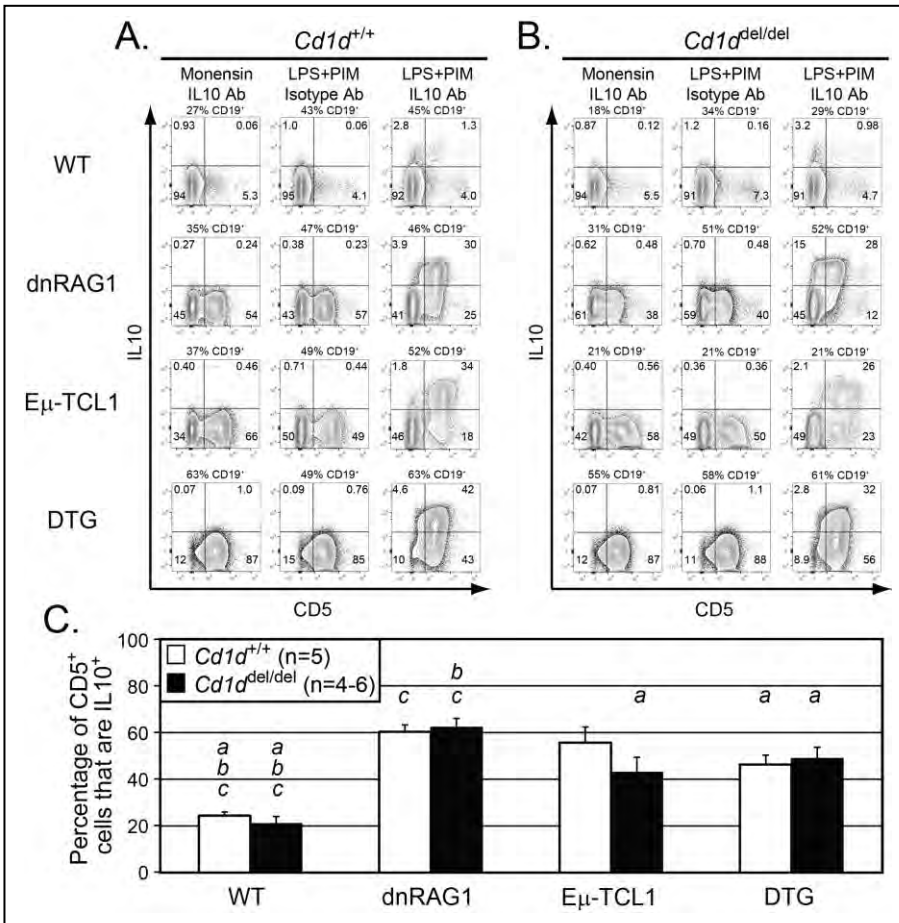


Figure 2. $CD5^+$ B cells in dnRAG1 and DTG mice support inducible IL10 expression *in vitro*, but IL10 competence is CD1d-independent. (A-B) Splenocytes from wild-type, dnRAG1, Eμ-TCL1, and DTG mice on a $Cd1d^{+/+}$ (A) or $Cd1d^{del/del}$ (B) background were incubated with monensin alone or LPS+PIM for 4 hrs. After permeabilization, the cells were stained with IL-10-specific or isotype-control monoclonal antibody (Ab) as indicated. Gated $CD19^+$ B cells were analyzed for CD5 and IL10 expression. The percentage of cells in each quadrant is shown for representative animals. (C) The mean percentage of total $CD5^+$ B cells staining positive for IL10 for n=4 mice/genotype is plotted in bar graph format. Error bars represent the SEM. Statistically significant (p<0.05) differences between groups of WT and transgenic animals with the same *Cd1d* genotype are indicated (*a* vs dnRAG1; *b* vs Eμ-TCL1; *c* vs DTG). No significant differences between $Cd1d^{+/+}$ and $Cd1d^{del/del}$ animals with the same transgenes were detected.

initiated experiments to evaluate CD5$^+$ B cell development and function and antibody levels in dnRAG1 mice in which IL10 has been knocked out.

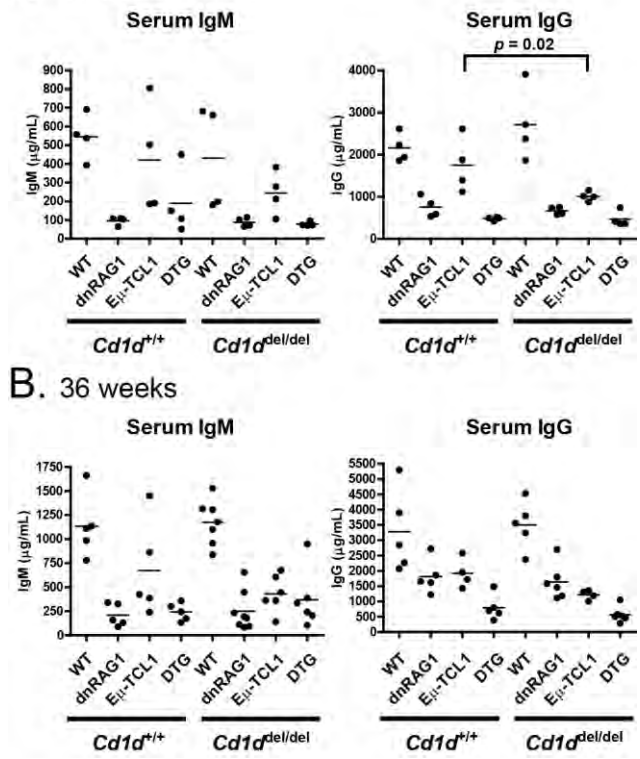


Figure 3. Loss of CD1d expression does not significantly affect spontaneous antibody production in dnRAG1 and DTG mice, but decreases spontaneous IgG production in Eμ-TCL1 mice. Serum IgM and IgG was measured from 12 (A) and 36 (B) week-old WT, dnRAG1, Eμ-TCL1, and DTG mice on a Cd1d$^{+/+}$ or Cd1d$^{del/del}$ background. Individual values and means (identified by horizontal bar) are shown for each group; statistically significant differences between groups are indicated.

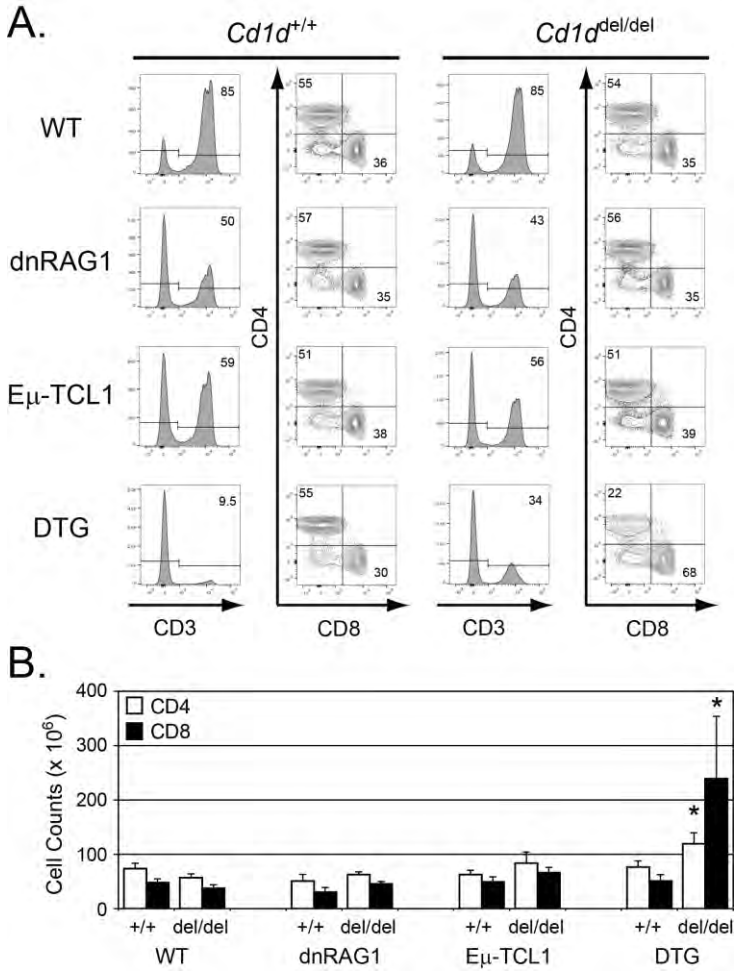


Figure 4. Loss of CD1d expression in 36 week-old DTG mice is associated expansion of splenic T cells, particularly CD8$^+$ T cells. (A) Gated splenic CD3$^+$B220$^-$ lymphocytes were analyzed for CD4 and CD8 expression. The percentage of cells within the identified gates or quadrants is shown for representative animals. Data collected from n=4-6 animals/genotype were used to calculate the absolute number of cells in each population. (B) Values for the mean absolute number of CD4$^+$ and CD8$^+$ T cells determined from (A) are plotted in bar graph format. Error bars represent the standard error of the mean. Statistically significant differences (p<0.05) between groups of *Cd1d*$^{+/+}$ or *Cd1d*$^{del/del}$ mice with the same transgenic background are identified by an asterisk.

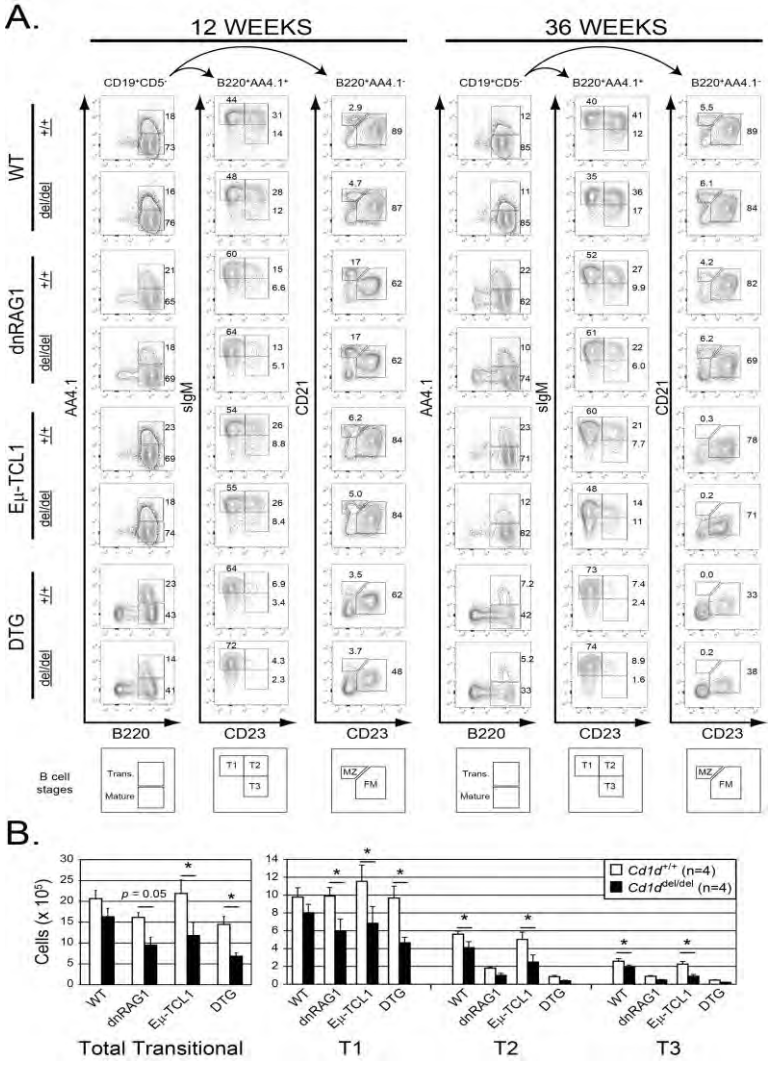


Figure 5. Progression through the transitional stages of B cell development is impaired in *Cd1d*$^{del/del}$ mice. (A) Gated splenic CD19^{+}CD5^{-} B cells from 12 week-old or 36 week-old (B) WT, dnRAG1, Eμ-TCL1 and DTG mice on a *Cd1d*$^{+/+}$ (+/+) or *Cd1d*$^{del/del}$ (del/del) background were initially segregated into transitional (trans.) and mature B cell subsets based on differential expression of AA4.1 and B220. Transitional (AA4.1^{+}B220^{hi}) and mature (AA4.1^{-}B220^{hi}) B cell subsets were further separated into T1-T3, marginal zone (MZ) and follicular mature (FM) populations based on differential expression of IgM, CD21, and CD23 using the gating strategy found at the bottom of the figure. The percentage of cells within the identified gates is shown for representative animals. Data collected from n=4-6 animals/genotype were used to calculate the absolute number of cells in each population. (B) Values for the mean absolute number of total transitional B cells (B220^{hi}AA4.1^{+}CD5^{-}) and for each transitional B cell subset (T1-T3) are plotted in bar graph format for the 12 week time point in (A). Error bars represent the standard error of the mean. Statistically significant differences (p<0.05) between groups of *Cd1d*$^{+/+}$ or *Cd1d*$^{del/del}$ mice with the same transgenic background are identified by an asterisk.

C. Significance

The findings in Aim 1 are significant because they represent the first formal demonstration that S100A6 is upregulated in human CLL at the protein level. These results suggest S100A6 may be a useful biomarker and/or target for therapeutic intervention in human CLL. The findings in Aims 2 and 3 are too preliminary to establish significance. The findings in Aim 4 are significant because they may have important implications for the potential success of therapeutic strategies targeting CD1d in CLL, because downregulation of CD1d as an evasion mechanism would be expected to have little or no effect on the continued survival and proliferation of the leukemic cells. On the other hand, the reduction of transitional B cells in CD1d-deficient transgenic mice raise the possibility that CD1d-restricted NKT cells play a previously unrecognized role in mediating tolerance induction in developing B cells reactive toward membrane-associated lipid antigens. The reduced numbers of splenic transitional B cells in transgenic mice bred onto a Cd1d-deficient background suggests the normal role for the CD1d-restricted NKT cells in this context is to provide pro-survival signals to transitional B cells reactive toward membrane-associated antigens, perhaps to facilitate receptor editing at the immature B cell stage of development to rescue B cell autoreactivity. Finally, The increase in splenic T cell numbers, particularly CD8^{+} T cells, in 36 week-old Cd1d-deficient DTG mice compared to Cd1d-proficient DTG mice was unexpected, and raises the possibility that CD1d expression and/or CD1d-restricted NKT cells regulate splenic T cell expansion in DTG mice during disease progression. Interestingly, reminiscent of Cd1d-deficient DTG mice, human CLL patients often show a paradoxical elevation in the absolute number of T cells, due mainly to an increase of CD8^{+} T cells (Mellstedt and Choudhury 2006). This observation has led to the suggestion that these

T cells foster an environment to promote leukemic cell growth or survival. The data presented here raise the possibility that in human CLL, NKT cells may be responsible for restraining T cell proliferation to control CLL progression, which would be consistent with reports correlating loss of NKT-like cells with disease progression in human CLL (Bojarska-Junak, et al 2010, Jadidi-Niaragh, et al 2012).

II. List of refereed publications germane to this project from 7/1/2014–6/30/2015

None yet, but a manuscript containing the data presented in Aim 4 was submitted to *British Journal of Haematology* on July 8, 2015.

III. List of extramural grants submitted from 7/1/2014–6/30/2015

Agency: NIH/NIAID R21 AI119829-01, submitted October 2014.
Role: PI
Title: Implications of B10-like cell expansion in a model of impaired receptor editing
Dates: 07/01/2015 to 06/30/2017
Amount: $400,125 total

Agency: NIH/NIGMS 3R01GM102487-03S1
Role: PI
Title: "A flow cytometer for multi-color cell analysis in a multi-user facility"
Dates: July 1, 2014 to June 30, 2015
Amount: $100,950

Agency: NIH/OD 1S10 OD021614-01
Role: PI
Title: "FACSAria Fusion Flow Cytometer"
Dates: February 1, 2016 to January 31, 2017
Amount: $701,190

Agency: Nebraska Center for Cellular Signaling funding by NIH/NIGMS, submitted May 2015
Title: "Determinants of the B10 phenotype in chronic lymphocytic leukemia (CLL)"
Dates: September 1, 2015 to August 31, 2016
Amount: $100,000

IV. List of extramural grants awarded from 7/1/2014–6/30/2015

Agency: NIH/NIGMS 5R01GM102487-04
Role: PI
Title: "Role of VprBP in B cell development and V(D)J recombination"
Dates: July 1, 2015 to June 30, 2016
Amount: $ 276,436

Agency: NIH/NIGMS 3R01GM102487-03S1
Role: PI
Title: "A flow cytometer for multi-color cell analysis in a multi-user facility"
Dates: July 1, 2014 to June 30, 2015
Amount: $100,950

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

MOLECULAR MECHANISMS AND TARGETS IN CANCER RESEARCH PROGRAM PROGRESS REPORT

Laura Hansen, PhD, Director

Project 4: Targeting Androgen Receptor and TRAIL: A Novel Treatment Paradigm for Breast Cancer

Principal Investigator: Yaping Tu, PhD

I. Progress Report Summary

A. Specific Aims

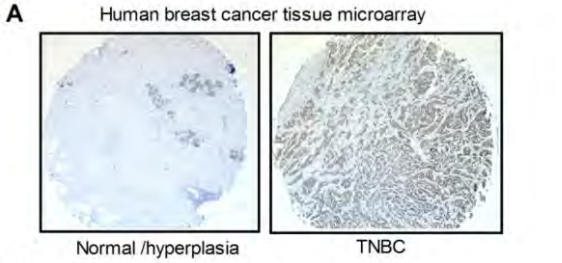
Aim 1: To assess the pathological importance of AR upregulation in TRAIL-resistant breast cancer progression.

Aim 2: To determine molecular mechanisms of AR suppression of TRAIL-signaling in breast cancer cells.

Aim 3: Targeting AR to improve the potency of TRAIL therapy.

B. Studies and Results

We made significant research progress in the past year. Based on the preliminary data, we submitted an R21 application to NIH and received an Impact/Priority Score of 25 (11%). One of the reviewer's major questions is when TRAIL therapy is recommended during the treatment of a breast cancer patient. Clinical trials of TRAIL for breast cancer have generally been disappointing because most breast cancer cells are resistant to TRAIL-induced apoptosis. However, a recent Phase II Trial showed that a combination of tigatuzumab (an agonistic anti-DR5 antibody, similar to TRAIL function) and chemotherapy prolonged progression-free survival in a few patients with metastatic triple-negative breast cancer (TNBC) (1). TNBCs (ER−/PR−/HER2−) are highly aggressive with poor prognosis and limited treatment options. Less than 30% of TNBC patients survive 5 years (2). Hence, there is an urgent need to develop safer and more effective treatments for TNBC (3). Thus, we revised the proposal and focused on TNBC.



B AR expression by immunohistochemistry staining in TNBC specimens

Breast specimens	n	Staining intensity 0	1	2	3	Average ± SE
Normal /hyperplasia	34	25	7	2	0	0.32 ± 0.10
TNBC	70	38	8	7	17	1.04 ± 0.14*

Statistical significance was determined using a Kruskal-Wallis test and Dunn posttest. *p<0.01 *vs*. Normal/hyperplasia.

Fig.1. AR upregulation in archival breast cancer specimens. (A) IHC staining for AR in normal/hyperplasia and TNBC. **(B)** Expression levels of AR protein were graded from 0 to 3 based on overall staining intensity.

1) AR is upregulated in archival human TNBC specimens. AR protein levels in breast tissue arrays (Biomax) were examined by IHC staining. **Fig.1A** shows that AR expression levels are markedly elevated in TNBC compared to normal or hyperplasic breast tissue. Only 26% of normal/hyperplastic tissues (9 out of 34) were AR-positive. In contrast, about 46% of TNBC (32 out of 70) were AR-positive. However, it should be noted that AR expression levels in these TNBC specimens varied significantly (**Fig.1B**).

2) Overexpression of AR in TNBC cell lines suppressed TRAIL-induced cell apoptosis. MDA-MB-231 and -436 are two TNBC cell lines. We previously showed that these two lines express very low levels of AR and are very sensitive to TRAIL-induced cell apoptosis compared to AR-positive breast cancer MCF-7 and T47D cells. Thus, we stably expressed

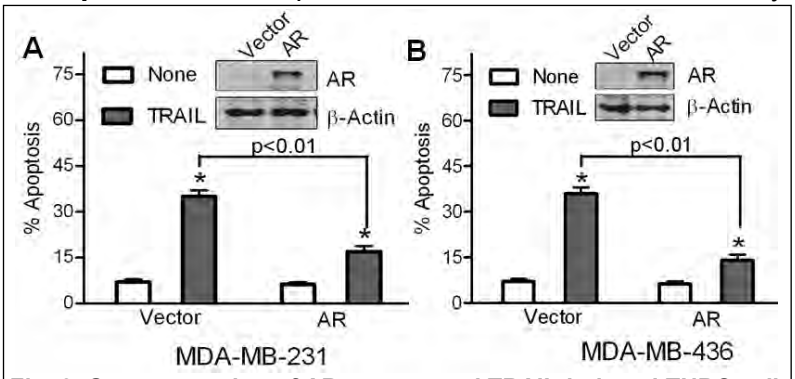


Fig. 2. Overexpression of AR suppressed TRAIL-induced TNBC cell apoptosis. MDA-MB-231 cells **(A)** and -436 cells **(B)** were stably transfected with AR and incubated with 50 ng/ml TRAIL for 6h. Inset, Western blot showing overexpression of exogenous AR. Apoptosis was evaluated by flow cytometric analysis following Annexin-V-FITC/propidium iodde (PI) staining. Data are expressed as % of Annexin V-FITC- and PI-negative cells (early stage of apoptosis) and represent the mean values of three independent experiments.

recombinant AR in MDA-MB-231 and -436 cells and found that overexpression of AR suppressed TRAIL-induced cell apoptosis (**Fig.2**). We will assess the effect of exogenous AR on TRAIL-induced regression of MDA-MB-231 tumors *in vivo*.

3) Knockdown of endogenous AR enhanced TRAIL-induced cell apoptosis. MDA-MB-453 is anaplastic-type, ER- and PR-negative breast cancer cell line with very low levels of HER2. It is perhaps the best-defined breast cancer cell line with regard to AR function (4,5). We found that this cell line expresses high levels of AR and is insensitive to TRAIL-induced cell apoptosis, similar to ER+/PR+/HER2+ MCF-7 cells. More importantly, knockdown of AR rendered both MDA-MB-453 (**Fig.3A**) and MCF-7 cells (**Fig.3B**) sensitive to TRAIL-induced apoptosis.

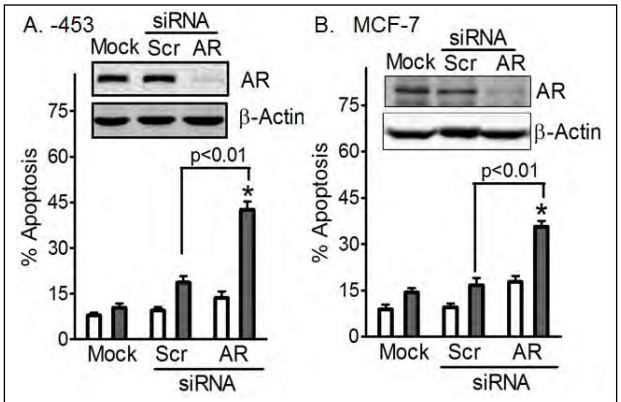


Fig. 3. Knockdown of AR enhanced TRAIL-induced breast cancer cell apoptosis. MDA-MB-453 cells **(A)** and MCF-7 cells **(B)** were transfected with scramble (Scr) or AR-specific siRNA. 72 h after transfection, AR expression levels were determined by Western blot assay (inset). Data are expressed as % of Annexin V-FITC- and PI-negative cells (early stage of apoptosis) and represent the mean values of three independent experiments. *p < 0.01

4) AR antagonist MDV3100 increases the TRAIL sensitivity of TNBC cells. MDA-MB-468 is a TRAIL-insensitive, AR-positive TNBC cell line (6). Treatment with MDV3100 (Enzalutamide), a new AR antagonist clinically used for castration-resistant prostate cancer treatment (7), enhanced TRAIL-induced T47D cell apoptosis (**Fig.4A**). MDV3100 also markedly enhanced TRAIL-induced MDA-MB-453 cell apoptosis (**Fig.4B**).

We are currently performing *in vitro* drug interaction experiments to evaluate the effect of various concentrations of MDV3100 on dose-dependent TRAIL-induced apoptosis of MDA-MB-468 cells. Dose-dependence curves for MDV3100 and TRAIL will be used to assess if the drug combination is additive or synergistic. The range of doses within which the drugs do interact will provide the basis for our *in vivo* study.

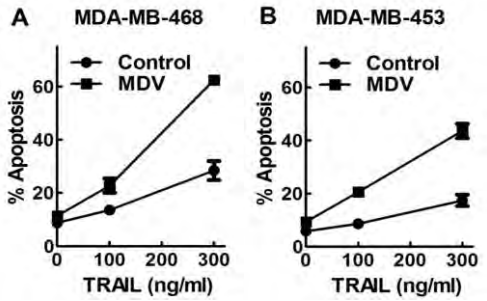


Fig.4. AR antagonist MDV3100 increased the TRAIL sensitivity of breast cancer cells. (A) Stimulation of TRAIL-induced apoptosis of MDA-MB-468 (**A**) and -453 cells in regular growth medium by MDV3100. Data are means ± S.E, n=3

Fig.5. Silence or inhibition of AR upregulates DR5 expression. (**A**) 50h after transfection with control or AR siRNAs, HeLa cell lysates were probed with the indicated antibodies. MDA-MB-468 (**B**) and T47D (**C**) cells were treated with or without 10μM MDV3100 or Casodex for 36h. DR5 levels were examined by Western blot. Images are representative of four experiments.

5) AR antagonist MDV3100 upregulates DR5 expression in breast cancer cells. Upon binding to its cell surface death receptor DR5, TRAIL induces the formation of the Death-Inducing Signaling Complex (DISC), which includes DR5, the adaptor molecule FADD, and pro-caspase 8. Through this proximity, caspase 8 is activated, and then activates the executioner caspases, such as caspase 3. We examined potential regulation of the DISC by the loss of AR using HeLa cells as a model because of their high transfection efficiency. While other DISC components did not show a change, DR5 was upregulated following siRNA knockdown of AR (**Fig.5A**). Treatment of T47D and MDA-MB-468 cells with AR antagonists Casodex or MDV3100 also upregulated the DR5 protein (**Fig. 5B and 5C**). These results suggest that AR inhibits the TRAIL-signaling by downregulating DR5.

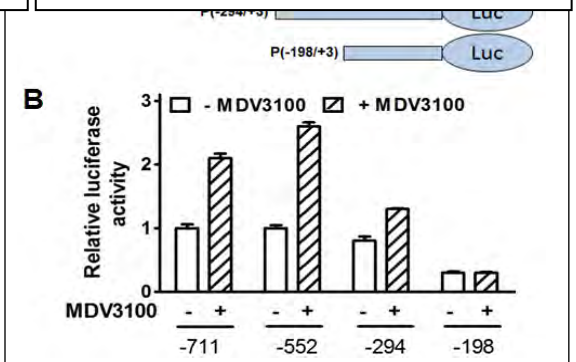


Fig. 6. Identification of a minimal promoter domain as critical for MDV3100-mediated activation of DR5 gene transcription. The promoter activity and MDV3100 response of pGL3 vectors containing various lengths of the DR5 promoter were assayed in MDA-MB-468 cells. Data shown are means ± S.E. of at least three independent experiments. **(A)** Serial deletion constructs from the 5′ site were created as shown. **(B)** Their relative promoter activities.

6) Cloning a proximal promoter of DR5 gene. AR was recently shown to act as a transcriptional repressor in prostate cancer cells (8-10). Thus we cloned a 714-bp fragment containing the proximal promoter of the DR5 gene from

MDA-MB-468 breast cancer cells and inserted it into the pGL3 luciferase reporter vector. A series of truncation mutants were also generated (**Fig. 6A**). The vectors were transfected into MDA-MB-468 breast cancer cells. Cells were treated with or without MDV3100 and luciferase activities were measured. As shown in **Fig. 6B**, DR5 promoter reporter constructs (-711/+3) and (-552/+3) had a 2-fold enhanced luciferase activity in cells treated with MDV3100 compared to that in control cells. The construct (-294/+3) had a reduced basal activity but was further enhanced by MDV3100 treatment. In contrast, the construct (-198/+3) had much lower basal activity and did not respond to MDV3100 treatment. Taken together, certain transcription factor binding motifs located between -294 and -198 may be responsible for the effect of MDV3100.

C. Significance

Triple-negative breast cancers (TNBC) are highly aggressive with poor prognosis. Treatment options for TNBC are mostly limited to chemotherapy that has life-threatening toxicities. Hence, there is an urgent need to develop new therapies for TNBC. TRAIL (TNF-related apoptosis-inducing ligand) is a promising cancer therapeutic agent due to its minimal toxicity to normal tissues and remarkable apoptotic activity in tumors. However, most breast cancer (BC) cells are resistant to TRAIL-induced apoptosis. Understanding the molecular mechanisms of TRAIL resistance and developing strategies to overcome such resistance is extremely important. Using a genomewide siRNA screening approach, we unexpectedly identified androgen receptor (AR) to be responsible for TRAIL resistance. In addition, we found that AR is markedly elevated in human invasive BC, including TNBC specimens. Importantly, human BC cell lines express different levels of AR that correlate with their TRAIL resistance, and suppressing AR sensitizes BC cells to TRAIL.

Successful completion of this project will identify functional molecules, either AR or other new targets in the AR signaling pathway, which may impact TRAIL sensitivity. Establishing the pathological role of this new AR signaling pathway in TRAIL resistance will also advance our knowledge of mechanisms of resistance to TRAIL-therapy and offer multiple novel drug targets as a first step to address the overarching challenge of developing effective treatments for TNBC. In fact, a recent Phase II trial showed that tigatuzumab (an agonistic anti-DR5 antibody, similar to TRAIL-therapy), in a combination with chemotherapy, had prolonged clinical benefit in some metastatic TNBC patients. Our studies could further provide a more accurate classification of TNBC that considers AR expression levels as a predictor of tumor response to TRAIL-therapy. This will provide rationales to design either TRAIL-monotherapy or combinational approaches of TRAIL with AR-directed therapy, which will speed progress towards our ultimate goal of tailoring a specific treatment plan for each TNBC patient. Since AR inhibitors are already used clinically for prostate cancer treatment, completion of our studies could have an immediate impact on TNBC patients. Moreover, the mechanisms unraveled here may also guide development of novel therapies for other types of TRAIL-resistant cancer with AR overexpression. Thus, our studies will have a significant impact on both basic and clinical cancer research.

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II. List of publications (7/1/2014 – 6/30/2015)

1. Zhou, T., Yu, M., Zhang, B., Wang, L., Wu, X., Zhou, H., Du, Y., **Tu, Y.,** Chen, C., Wei, T. (2014) Inhibition of cancer cell migration by gold nanorods: molecular mechanisms and implications for cancer therapy. *Advanced Functional Materials* 24: 6922–6932 **(IF: 11.805)**
2. Zhou, T., Zhang, B., Wei, P., Du, Y., Zhou, H., Yu, M, Yan, L, Zhang, W, Nie, G., Chen, C., **Tu, Y.**, Wei, T. (2014) Energy metabolism analysis reveals the mechanism of inhibition of breast cancer cell metastasis by PEG-modified graphene oxide nanosheets. *Biomaterials* 35: 9833-9843. PMID: 25212524 **(IF: 8.557)**
3. Barrio-Real, L., Benedetti, L.G., Engel, N., **Tu, Y.**, Cho, S., Sukumar, S., Kazanietz, M.G. (2014) Subtype-specific overexpression of the Rac-GEF P-REX1 in breast cancer is associated with promoter hypomethylation. *Breast Cancer Res1*6:441. PMCID: PMC4303123 **(IF: 5.49)**
4. Jiang, H., Xie, Y., Abel, P.W., Wolff, D.W., Toews, M.L., Panettieri. Jr R.A., Casale, T.B., **Tu, Y.** (2015) RGS2 repression exacerbates airway hyperresponsiveness and remodeling in asthma. *Am J Respir Cell Mol Biol* 53:42-49. PMID:25368964 **(IF: 4.11)**

III. List of extramural grants submitted from 7/1/2014 – 6/30/2015

NIH – R21
Title: Androgen Receptor: A Key Regulator of TRAIL Resistance in Breast Cancer?
Dates: 1/2015 - 12/2016
PI: Yaping Tu
Total funds requested: $418,375
Impact score 25 (11%): pending

DoD Breast Cancer Breakthrough Award Levels 1 and 2
Title: Targeting Androgen Receptor and TRAIL: A Novel Treatment Paradigm for Breast Cancer
Dates: 1/1/2016 - 12/31/2018
PI: Yaping Tu
Total funds requested: $541,875.00
Under review

NIH – R01
Title: Targeting Gia2 in Metastatic Prostate Cancers
Dates: 4/1/2016 - 3/31/2021
Co-PI: Yaping Tu
Total funds of sub-award requested: $454,688
Under review

NIH – R21 (resubmission)
Title: Androgen Receptor: A Key Regulator of TRAIL Resistance in Breast Cancer?
Dates: 4/1/2016 - 3/31/2018
PI: Yaping Tu
Total funds requested: $418,375
Under review

IV. List of extramural grants awarded from 7/1/2014 – 6/30/2015

Department of Defense Prostate Cancer Research Program – Idea Award
Title: Aberrantly Upregulated P-Rex1 Promotes Castration-Resistant Prostate Cancer Progression
Dates: 7/2013 - 6/2016
PI: Yaping Tu
Award: $541,875

National Institutes of Health - NHLBI R01HL116849

Title: Dysregulation of RGS2 Protein and Airway Hyperresponsiveness in Asthma

Dates: 8/2013 – 7/2017
PI: Yaping Tu
Award: $1,437,540

Department of Defense Prostate Cancer Research Program – Hypothesis Award
Title: LincRNAs and AR Reactivation after Androgen-Deprivation in Prostate Cancer Cells
Dates: 10/2013 - 9/2014
PI: Xianming Chen
Role: Co-Investigator (5%)
Sub-Award: $9,100

National Institutes of Health, 5P20GM103489
Title: Nebraska Center for Cellular Signaling
Dates: 7/2013-6/2018
Collaborative Project:
Title: PDE4 in lung disease
Dates: 7/2014-6/2015
PI: Keith Johnson
Role: Co-Investigator (1%)
Total funds requested: $100,000
Sub-Award: $2,000

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

MOLECULAR AND CELLULAR MECHANISMS OF SMOKING-RELATED LUNG DISEASE RESEARCH PROGRAM PROGRESS REPORT

Yaping Tu, PhD, Director

Project 1: P-Rex1 Repression and Early-Life Environmental Cigarette Smoke-Increased Risk of Asthma

Principal Investigator: Yaping Tu, PhD

I. Progress Report Summary

A. Specific Aims

- Aim 1: To determine the mechanism by which P-Rex1 regulates ASM innervation.
- Aim 2: To elucidate the mechanism of IL-6-induced repression of neuronal P-Rex1.
- Aim 3: To investigate the pathologic importance of P-Rex1 repression in pulmonary neurons *in vivo*.

B. Studies and Results

We made some research progress in the past year. Since P-Rex1 is highly expressed in neuronal cells but very low in airway smooth muscle (ASM) cells, we initially focused on neuronal P-Rex1 regulation of ASM innervation. Our data suggested that P-Rex1 may suppress neurite growth and extension.

1. Loss of P-Rex1 enhanced neurotrophic factor-stimulated neurite outgrowth and extension of mouse spinal cord neurons. Previous studies showed that ASM-derived neurotrophic factors induce neural innervation of ASM. Indeed, conditioned medium (CM) derived from human ASM cells stimulated neurite growth and extension of mouse spinal cord neurons (**Fig. 1,** A *vs*. C). Interestingly, loss of P-Rex1 increased ASM CM stimulatory effects (**Fig.1**, C vs. D). Loss of P-Rex1 also enhanced neurotrophic factor NGF-stimulated neurite outgrowth of mouse spinal cord neurons (**Fig.1E**).

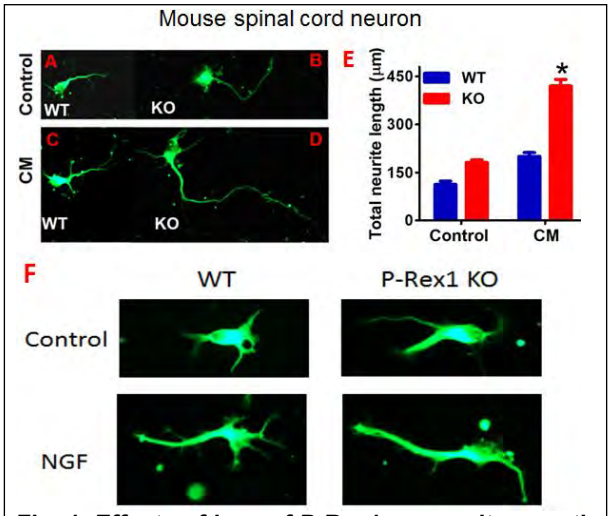

Fig. 1. Effects of loss of P-Rex1 on neurite growth and extension. One-day *in vitro* (DIV) spinal cord neurons isolated from WT and P-Rex1 KO embryonic mice were treated with or without ASM CM (A-E) or NGF (25 ng/ml) for 24h. Neurons were immunostained for β-tubulin. (**A-D, F**) Representative images of ASM CM-induced neurite growth (Scale bar: 50 μm.) (**E**) Quantification of neurite growth. Each value represents mean ± SE of 50 neurons.

2. Knockdown of endogenous P-Rex1 increase NGF-stimulated neurite outgrowth of rat neuron-like PC12 cells. Since the rat PC12 cell line is extensively used as *in vitro* models to study the mechanisms associated with neurotrophin-induced neurite outgrowth, we used PC12 cells to further investigate the importance of P-Rex1 in regulation of NGF-stimulated neurite outgrowth. As shown in **Fig. 2**, NGF treatment induced neurite outgrowth in PC12 cells. P-Rex1-specific siRNA reduced endogenous P-Rex1 expression in PC12 by more than 90%, which further enhanced NGF-stimulated neurite outgrowth.

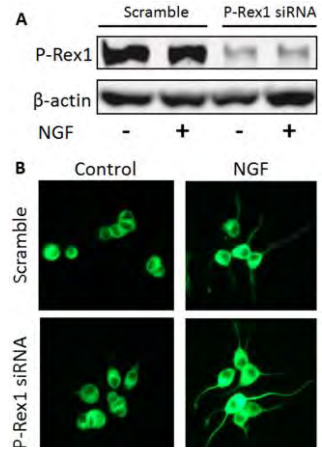

Fig.2. Knockdown of P-Rex1 increases neurite outgrowth of PC12 cells stimulated by NGF. PC12 cells were transfected with scramble or P-Rex1-specific siRNA for 48 h. Cells were treated with or without NGF (25 ng/ml) for 24 h and then were subjected to western blot assays of P-Rex1 protein **(A)** or immunostained for β-tubulin **(B).** Images shown are representative of 3 independent experiments

3. Expression of exogenous P-Rex1 attenuated IL-6 stimulatory effects on NGF-induced neurite growth. Cigarette smoking causes airway inflammation characterized by increased concentrations of cytokines, such as TNF-α, IL-6, and IL-8, in humans and animals. IL-6 is thought to play a critical role in the pathogenesis of lung diseases. However, the effects and mechanisms of IL-6 modulation of airway innervation are unknown. In our preliminary studies, we found that

IL-6 enhanced ASM CM-stimulated neurite growth of WT but not P-Rex1 KO mouse spinal cord neurons (data not shown).

Interestingly, treatment with IL-6 downregulated P-Rex1 protein expression by about 50% and stimulated NGF-induced neurite outgrowth of PC12 cells (**Fig.3**). Thus, we determined whether expression of exogenous P-Rex1 can attenuate the stimulatory effects of IL-6 on neurite growth. As shown in **Fig.4**, IL-6 treatment decreased endogenous P-Rex1 expression and significantly increased NGF-stimulated neurite outgrowth of PC12 cells. Overexpression of exogenous P-Rex1 attenuated neurite growth of PC12 cells induced by IL6 and NGF treatment.

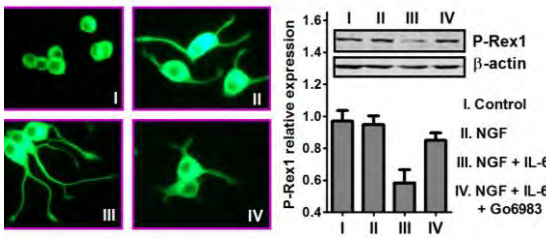

Fig. 3. IL-6 downregulates P-Rex1 and enhanced NGF-induced neurite outgrowth of PC12 cells. Cells were treated with β-NGF (25 ng/ml) with or without IL-6 (50 ng/ml) in the absence or presence of PKC inhibitor Go6983 (2 μM) for 36 h. (**A**) Images of neurite outgrowth of PC12 cells immunostained with a β-tubulin antibody. (**B**) Western blot for P-Rex1.

4. PKC activation is involved in IL-6-promoted P-Rex1 repression and neurite growth. We have found that effects of IL-6 on P-Rex1 protein expression and neurite outgrowth of PC12 cells could be attenuated by pre-treatment with the general PKC inhibitor Go6983 (see **Fig.3**), suggesting the importance of PKC signaling pathways.

5. PKC activator phorbol 12-myristate 13-acetate (PMA) augments NGF-stimulated P-Rex1 repression and neurite growth. We stimulated PC12 cells with NGF in the presence or absence of PKC activator phorbol 12-myristate 13-acetate (PMA). As shown in **Fig. 5**, PMA treatment enhanced NGF-induced P-Rex1 repression and neurite growth of PC12 cells, which was attenuated by pre-treatment with Go6983.

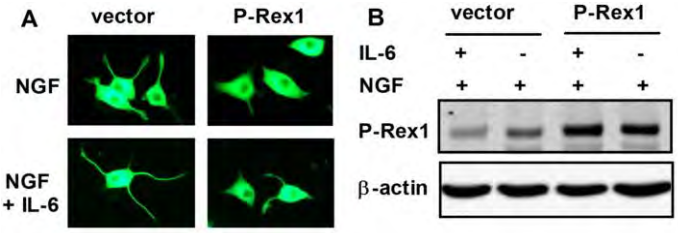

Fig.4. Expression of exogenous P-Rex1 attenuated IL-6 stimulatory effects on NGF-induced neurite growth of PC12 cells. Cells were transfected with control vector or vector expressing P-Rex1 for 24 h. Cells were treated with or without NGF (25 ng/ml) for 24 h and then were subjected to β-tubulin immunostaining **(A)** and western blot assays of P-Rex1 **(B)**.

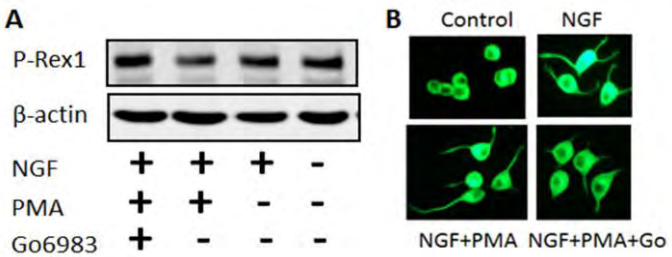

Fig.5. PMA augments NGF-stimulated P-Rex1 repression and neurite growth. Cells were treated with or without Go6983 (2μM) for 30 min and then stimulated with or without NGF (25 ng/ml) for 24 h in the absence or presence of PMA (50 nM). Cells were subjected to western blot assays of P-Rex1 **(A)** and β-tubulin immunostaining **(B).**

Expansion of the project: Dysregulation of P-Rex1 and ASM Remodeling in Severe Asthma

We also obtained several primary human ASM cell lines from Dr. Rey Panettieri's lab, which were isolated postmortem from tracheal or bronchial smooth muscles of non-asthmatic and asthmatic donors who had fatal asthma. P-Rex1 expression levels in these cell lines were compared.

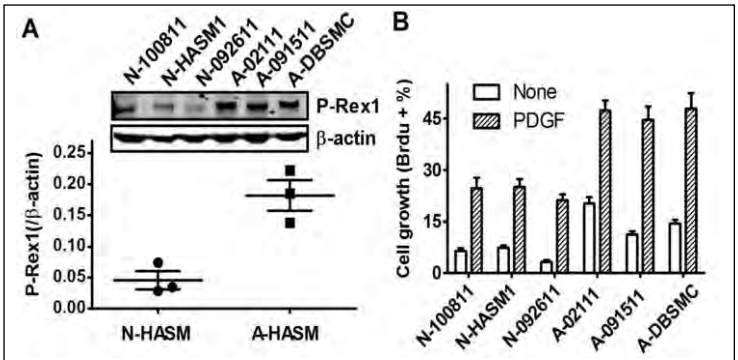

Fig. 6. Upregulation of P-Rex1 protein and increased proliferation of human asthmatic ASM cells. (A) P-Rex1 protein expression in N-HASM-N (n=3) and A-HASM cell lines (n=3) (Western blot), quantitated by densitometry, normalized by β-actin. **(B)** 5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay. Cells were treated with or without PDGF (10 ng/ml) for 24 h. The results were expressed as the percentage of BrdU positive cells to total DAPI stained cells.

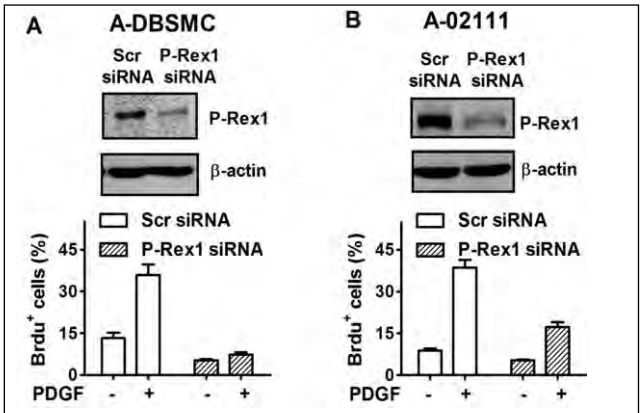

Fig. 7. P-Rex1 is required for mitogen PDGF-stimulated A-HASM cell proliferation. A-DBSMC **(A)** and A-02111 **(B)** cells were transfected with P-Rex1-specific siRNA or a scrambled siRNA. P-Rex1 expression levels were determined by western blot. Cell proliferation was measured by BrdU assays.

6) Upregulation of P-Rex1 protein and increased proliferation of human asthmatic ASM cells. Western blot showed that human non-asthmatic ASM (N-HASM) cells express P-Rex1 protein but at low levels. Human asthmatic ASM (A-HASM) cell lines (n=3) expressed 3.5-fold higher P-Rex1 protein than N-HASM cell lines (n=3) (**Fig.6A,** $p<0.01$). Interestingly, A-HASM cell lines proliferate by 2-fold faster than N-HASM cell lines in the absence or presence of mitogen PDGF (**Fig. 6B**).

7) P-Rex1 is required for mitogen PDGF-stimulated A-HASM cell proliferation. We next addressed whether P-Rex1 upregulation modulates ASM proliferation. We silenced P-Rex1 in two A-HASM cell lines using siRNA and measured proliferation in the presence and absence of PDGF. P-Rex1 expression levels were reduced 60-70% in cells transfected with P-Rex1 specific siRNA relative to control cells with a scrambled siRNA. PDGF-stimulated proliferation was profoundly reduced in P-Rex1-depleted cells compared to control cells (**Fig.7**).

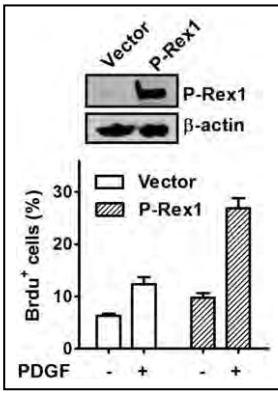

Fig.8. Effects of P-Rex1 overexpression on PDGF-stimulated N-HASM cell proliferation. N-HASM cells were transfected with control vector or vector expressing HA-tagged P-Rex1 for 24 h. Cells were treated with or without PDGF (10 ng/ml) for 24 h. Cell proliferation was measured by BrdU assays. Bars represents mean ± SE of three separate experiments.

8) P-Rex1 overexpression enhanced N-HASM cell proliferation. We also examined the effects of exogenously expressed HA-tagged P-Rex1 on proliferation of N-HASM cells. As shown in **Fig. 8**, P-Rex1 expression increased N-HASM cell proliferation by 2.5-fold in the absence or presence of mitogen PDGF.

C. Significance

Cigarette smoking is the most important source of preventable morbidity and premature mortality worldwide. Exposure to passive cigarette smoke from parents is also associated with lung disease in children. However, little is known about the molecular mechanisms of early-life environmental cigarette smoke (ECS) exposure-related lung disease. Our preliminary studies suggest a critical role of neuronal P-Rex1 in regulation of airway innervation and airway hyperresponsiveness (AHR), the pathophysiologic hallmark of asthma. Our immediate objective is to define the mechanism and pathological importance of neuronal P-Rex1 dysregulation in the development of early-life ECS exposure-related AHR. The data generated in the past year showed that loss of P-Rex1 enhanced neurotrophic factor-stimulated neurite outgrowth and extension of mouse spinal cord neurons and rat neuron-like PC12 cells. It provides experimental evidence supporting our previous observation that P-Rex1 knockout (KO) mice exhibit airway hyperinnervation and increased lung resistance. More importantly, we found that cigarette smoke-related inflammatory cytokine IL-6 downregulated P-Rex1 protein expression and stimulated NGF-induced neurite outgrowth of PC12 cells, which could be attenuated by expression of exogenous P-Rex1 or PKC inhibitor. Our studies provide a new mechanism underlying the early-life ECS exposure-induced airway hyperinnervation, which should have a significant impact on our basic knowledge of the molecules and mechanisms involved in the development and severity of cigarette smoke-

related asthma. Further studies to identify PKC isoforms that mediate IL-6-promoted P-Rex1 repression and neurite growth could guide development of novel therapeutics for patients with cigarette smoke-related asthma.

Despite intensive research efforts, 20% of patients with asthma develop irreversible airway obstruction, which is refractory to steroids and bronchodilators, and disproportionately accounts for asthma morbidity and mortality. Accumulating evidence suggests that cell proliferation contributes to airway smooth muscle hyperplasia and remodeling that lead to irreversible obstruction of airways in patients with severe asthma. However, the molecular mechanisms regulating the proliferation of HASM cells in asthma-related conditions are still poorly understood. Our new data suggest that dysregulated P-Rex1 may have a novel and critical role in development of severe asthma. Our studies will identify specific aberrant signaling pathways and molecules likely to be important in the enhanced ASM hyperplasia and remodeling in patients with severe asthma, leading to increased understanding of asthma pathogenesis, which could lead to development of specific therapies for treatment of severe asthma.

II. List of publications (7/1/2014 – 6/30/2015)

1. Zhou, T., Yu, M., Zhang, B., Wang, L., Wu, X., Zhou, H., Du, Y., **Tu, Y.,** Chen, C., Wei, T. (2014) Inhibition of cancer cell migration by gold nanorods: molecular mechanisms and implications for cancer therapy. *Advanced Functional Materials* 24: 6922–6932 **(IF: 11.805)**
2. Zhou, T., Zhang, B., Wei, P., Du, Y., Zhou, H., Yu, M, Yan, L, Zhang, W, Nie, G., Chen, C., **Tu, Y.**, Wei, T. (2014) Energy metabolism analysis reveals the mechanism of inhibition of breast cancer cell metastasis by PEG-modified graphene oxide nanosheets. *Biomaterials* 35: 9833-9843. PMID: 25212524 **(IF: 8.557)**
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III. List of extramural grants submitted from 7/1/2014 – 6/30/2015

NIH – R21
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Dates: 1/2015 - 12/2016
PI: Yaping Tu
Total funds requested: $418,375
Impact score 25 (11%): pending

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PI: Yaping Tu
Total funds requested: $541,875
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Dates: 7/2013 - 6/2016
PI: Yaping Tu
Award: $541,875

National Institutes of Health - NHLBI R01HL116849
Title: Dysregulation of RGS2 protein and airway hyperresponsiveness in asthma
Dates: 8/2013 – 7/2017
PI: Yaping Tu
Award: $1,437,540

Department of Defense Prostate Cancer Research Program – Hypothesis Award
Title: LincRNAs and AR reactivation after androgen-deprivation in prostate cancer cells
Dates: 10/2013 - 9/2014
PI: Xian-Ming Chen
Role: Co-Investigator (5%)
Sub-Award: $9,100

National Institutes of Health, 5P20GM103489
Title: Nebraska Center for Cellular Signaling
Dates: 7/2013-6/2018
Collaborative Project Dates: 7/2014-6/2015
Title: PDE4 in lung disease
PI: Keith Johnson
Role: Co-Investigator (1%)
Total funds requested: $100,000
Sub-Award: $2,000

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

MOLECULAR AND CELLULAR MECHANISMS OF SMOKING-RELATED LUNG DISEASE RESEARCH PROGRAM PROGRESS REPORT

Yaping Tu, PhD, Director

Project 2: Mollecular and Cellular Triggers of Early-Life Environmental Cigarette Smoke-Related Pulmonary Hypertension

Principal Investigator: Peter Abel, PhD

I. Progress Report Summary

A. Specific Aims

The specific aims of this project are listed below. These specific aims have not been modified.

- Specific Aim 1. To determine the role of early-life environmental cigarette smoke (ECS)-induced pulmonary vascular inflammation in causing pulmonary vascular remodeling and hyper-contraction in mice.
- Specific Aim 2. To test whether early life ECS-mediated hyper-innervation causes sensitization to pulmonary vascular remodeling and hyper-contraction produced by a second vascular insult in adult mice.
- Specific Aim 3. To examine the mechanisms of pulmonary vascular remodeling and hyper-contraction caused by early-life ECS followed by a second vascular insult in adult mice.

B. Studies and Results

The central hypothesis of this project is that early-life ECS causes inflammation related hyper-innervation and sensitization of the pulmonary vasculature to vascular insults in adulthood, causing enhanced pulmonary vascular constriction and leading to pulmonary hypertension (PH). The key features of this proposal are the use of ECS and P-Rex1 KO mice to produce pulmonary autonomic nerve hyper-innervation leading to development of PH. Our studies to date have focused on these key components of our proposal.

ECS Exposure

Specific aims 1, 2, and 3 rely on reliable and accurate animal exposure to ECS. Therefore, we have rebuilt and tested our Teague TE-10 smoking machine. After significant time and effort, we found that this machine was unable to reliably deliver reproducible ECS exposure as measured by total suspended particulates (TSP).

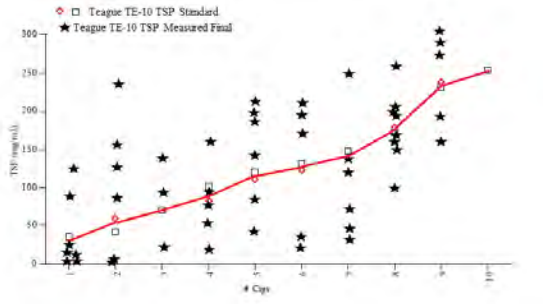
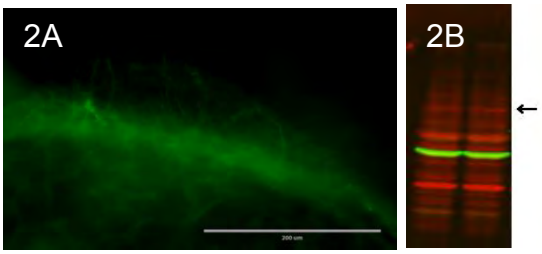


Fig. 1. Each point represents TSP measurements reported by Teague (box, red triangles, red line) compared with measurements made in our lab (stars). The amount of TSP delivered per cigarette varied widely. Because this variability was unacceptable, we have purchased a DSI Buxco smoking machine to replace the Teague TE-10.

Pulmonary Nerves

Identification of pulmonary vascular nerves - Specific Aim 1

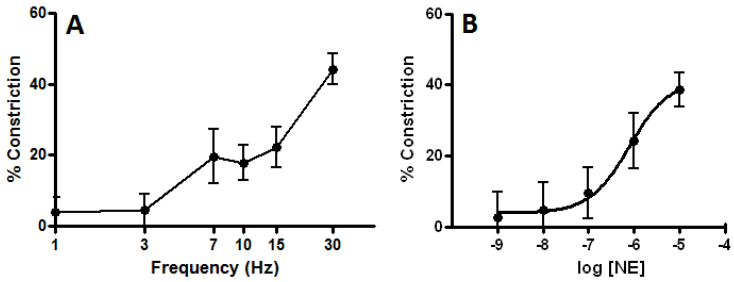
We performed immunohistochemistry (IHC) for total neurons in mouse lungs. Mouse lungs were inflated, fixed, sliced at 200 µm, and incubated with rabbit anti-beta III tubulin antibody (1:500, Aviva Bio Systems) to label total nerves. Slices were imaged using a fluorescent Zeiss Axioskop 2 microscope. A representative pulmonary artery surrounded by green beta III tubulin fluorescence is shown In Fig 2A. However, the images did not have sufficient precision to quantify nerve density. Western blotting suggests this antibody lacks sufficient specificity (Fig 2B). Arrow indicates beta III tubulin band size. Future IHC experiments will use the antibody and method described by Aven et al., who



have quantified nerves in mouse lungs.

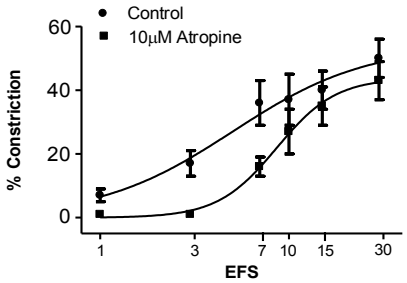
Function and neurotransmitter phenotype of pulmonary vascular nerves - Specific Aims 1, 2

Mouse precision-cut lung slices (PCLS) were prepared and frequency-response curves for vasoconstriction in response to electric field stimulation (EFS) were generated (Fig 3). Increasing frequencies produced increased pulmonary arterial vasoconstriction (Fig 3A) and dose-response curves for norepinephrine (NE) (Fig 3A) produced a similar effect. These effects were partially blocked by the α_1-adrenergic receptor antagonist prazosin, consistent with NE release from sympathetic nerves and causing constriction of mouse pulmonary arteries.



Function and neurotransmitter phenotype of airway nerves - Specific Aims 1, 2

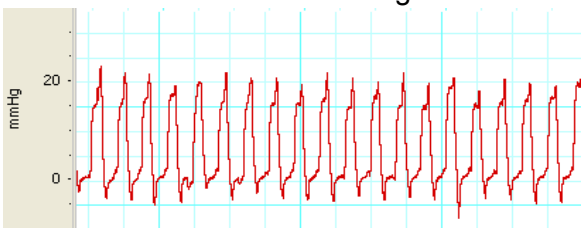
Mouse PCLS were prepared and frequency-response curves for bronchoconstriction or airways in response to EFS were also generated (Fig 4). As expected, increasing frequencies produced bronchoconstriction; however, atropine blocked the effects of low frequency but not high frequency stimulation. Thus, acetylcholine appears to be released from parasympathetic nerves at low frequencies, but a different neurotransmitter mediates bronchoconstriction at high frequencies.



Translational Applications

Measurement of pulmonary hypertension in mice – Specific Aim 3

We had proposed to use the Fulton Index (Weight of Right Ventricle / [Left Ventricle + Septum]) as an indirect measure of pulmonary hypertension (PH). This method has significant limitations; therefore, we have developed a method to quantify right ventricular pressure as a surrogate measurement for pulmonary arterial pressure. A Millar pressure catheter was inserted into the jugular vein and advanced into the right ventricle. A recording with the characteristic right ventricular pressure waveform and pressure range is shown in Fig. 5. This method will now be included in studies using intact animals to measure pulmonary hypertension.



Autonomic nerve function measurement in mice – Specific Aim 3.

We have identified and tested heart rate variability (HRV) as a potential measurement of changes in sympathetic and parasympathetic activity in live, intact mice. Wild-type and P-Rex1 KO mice were anesthetized, the ECG recorded, and the R-R interval calculated. HRV, the beat-to-beat variability of heart rate, was evaluated using fast Fourier transform and Poincare plots. Only preliminary data for sympathetic nerve activity is shown in the table: data for parasympathetic nerve activity is currently being evaluated. The heart rate was similar between wild-type and P-Rex1 KO mice. The ratio of low-frequency HRV power to high-frequency HRV power (FFT LH/HF) is a measure of sympathetic nerve activity. This result suggests that there is increased cardiovascular sympathetic nerve input in the hyper-innervated P-Rex1 KO mice. HRV is being evaluated as a measure of increased autonomic nerve activity in P-Rex1 KO mice and ECS-exposed mice, and as a novel translational marker for autonomic nerve activity in humans with PH or other lung diseases.

Strain	Mean Heart Rate	Standard Deviation	FFT LF/HF	Standard Deviation	N
P-Rex1 KO	342	10	1.8	11	11
Wild-Type	350	18	0.8	20	16

C. Significance

Cigarette smoking is the most preventable cause of premature death in the United States; cigarette smokers and those exposed to secondhand or ECS have a higher risk of developing chronic cardiovascular and respiratory diseases. COPD, emphysema, chronic bronchitis, pulmonary fibrosis and other hypoxia related lung diseases can all contribute to development of pulmonary hypertension (PH), and hypoxia and cigarette smoke are associated with PH in both humans and in animal models. There is a strong association between ECS and cardiovascular-related disability and death, and even short-term exposure to ECS can cause pulmonary vascular dysfunction and PH. Thus the link between smoking, respiratory disease, and cardiovascular disease, including PH, is strong.

The overall goal of this project is to identify mechanisms underlying ECS-induced pulmonary vascular dysfunction. We will integrate cellular and molecular studies with animal models to test the novel hypothesis that early-life ECS causes pulmonary vascular hyper-innervation, leading to phenotypic changes of the pulmonary vasculature that contribute to the development of pulmonary hypertension. Our objective is to define the pathological importance of pulmonary vascular inflammation and hyper-innervation in the development of early-life ECS sensitization to vascular insults leading to pulmonary vascular dysfunction in adulthood.

A possible new translational aspect of this work involves the application of heart rate variability as a potential marker for pulmonary hypertension in humans. The ease of heart rate measurements using common smart phone apps or consumer exercise monitors (e.g., Fitbit) provides feasibility for broad application for this marker.

II. Refereed publications germane to this project from 7/1/2014–6/30/2015

None

III. List of extramural grants submitted from 7/1/2014–6/30/2015

Agency: Citizens United for Research in Epilepsy
Title: *Is orexin a critical regulator of cardio-respiratory dysfunction causing SUDEP?*
Role: Co-investigator

Agency: American Heart Association
Title: *Pulmonary vascular hyper-innervation: A novel contributor to Pulmonary Hypertension or*
Role: Research Supervisor – Student Fellowship Grant

IV. List of extramural grants awarded from 7/1/2014–6/30/2015

None

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

MOLECULAR AND CELLULAR MECHANISMS OF SMOKING-RELATED LUNG DISEASE RESEARCH PROGRAM PROGRESS REPORT
Yaping Tu, PhD, Director

Project 3: Targeting the PKG Signaling Pathway in Early-Life Environmental Cigarette Smoke-Related Lung Disease
Principal Investigator: Peter Oldenburg, PhD

I. Progress Report Summary
Since obtaining this funding, we have been actively using pharmacological agents in precision-cut lung slices from mice to test how they affect the NO/cGMP/PKG signaling pathway. We have been unable to explore this pathway in mice exposed to cigarette smoke due to the proposed Teague TE-10 smoking machine being inoperable. We have remedied this issue by purchasing a new smoke-generating system from Buxco Electronics and will now be able to expose mice to cigarette smoke.

A. Specific Aims
The specific aims have not been modified. We hypothesize that early-life environmental cigarette smoke (ECS) exposure causes PKG-dependent pathological changes of airway smooth muscle in the lung, leading to AHR.

- **Aim 1:** Characterize how hyper-innervation as a result of early-life ECS exposure affects the NO/cGMP/PKG pathway in airway smooth muscle, leading to the AHR phenotype.
- **Aim 2**: Identify the role and mechanisms of PKG signaling in regulation of early-life ECS exposure-induced mouse airway and vascular hyperresponsiveness following a secondary allergen insult in mice.
- **Aim 3:** Characterize the effects of activation of the NO/cGMP/PKG pathway on airway and pulmonary vascular responsiveness in an *in vivo* early-life ECS exposure mouse model.

B. Studies and Results
Aim 1 Progress: In June 2015, we received and installed our new smoke generator system from Buxco Electronics and will now be able to expose mice to cigarette smoke. We have detected PKG by Western blotting and are working to optimize our protocol to yield stronger signals. We are working to detect guanylyl cyclase by Western blotting as well. Once mice have been exposed to smoke, we will measure their exhaled nitric oxide (NO) levels using the NIOX instrument. Following smoke exposure, we will then detect changes in PKG and guanylyl cyclase protein levels by Western blotting to determine the impact of early-life smoke exposure, as well as what effects early-life smoke exposure has on NO levels in mice.

Aim 2 Progress: We have made great progress in this aim. We have used the mouse precision-cut lung slice (PCLS) model to detect changes in the NO/cGMP/PKG signaling pathway after activation or inhibition with specific pharmacological agents that target this pathway. We are able to stimulate airway contraction using methacholine. We can block the nitric oxide synthase using L-NMMA and subsequently block the production and relaxing effects of NO. This blockade can be bypassed by use of sodium nitroprusside (SNP), which works

downstream of nitric oxide synthase (NOS) and activates guanylyl cyclase through its actions as a NO donor. Blocking guanylyl cyclase with the compound ODQ resulted in an inhibition of SNP-mediated relaxation. Pretreatment of PCLS with the guanylyl cyclase stimulator YC-1 resulted in an attenuation of methacholine-induced airway contraction. Use of the nonspecific phosphodiesterase inhibitor IBMX allowed guanylyl cyclase to remain active for a prolonged period of time, which resulted in airway relaxation. Inhibition of PKG using KT5823 blocked the ability of SNP to relax the airways during methacholine stimulation, demonstrating the importance of PKG activation in promoting dilation of the airways, especially during events that may induce severe constriction. In the next grant year, we will utilize the new smoke machine to identify how these compounds will affect the airways of mice that have been exposed to cigarette smoke.

Aim 3 Progress: Progress in this aim will be contingent on the positive results that we are observing in the PCLS being treated with pharmacological agents that activate the NO/PKG signaling pathway. Agents that activate this signaling pathway have demonstrated less airway constriction in the PCLS model, as described in aim 2, and give us a positive outlook as to how they will function *in vivo*. Since we have been unable to expose mice to cigarette smoke thus far, we have not administered any of these compounds to mice to observe their therapeutic effect on smoke-induced AHR. We originally proposed to begin these studies in year 3 and are confident that we will observe less AHR in the smoke-exposed mice following treatment with these agents and the data will correlate well with those that have been collected in the PCLS model.

C. Significance

There is strong evidence that parental smoking has adverse effects on the respiratory health of children and results in increased incidences of cancers, lung disease and altered airway reactivity. This is a major health issue because these children later develop lung diseases, which worsen when exposed to a secondary insult, such as cigarette smoke or allergens. Our studies will focus on whether targeting the NO/cGMP/PKG signaling pathway could ameliorate early-life ECS exposure-induced airway and pulmonary vascular responsiveness. Successful completion of our studies may lead to the development of specific therapies for early-life ECS-related lung diseases.

II. Publications germane to this project from 7/1/2014-6/30/2015.

None

III. List of extramural grants submitted from 7/1/2014-6/30/2015.

None

IV. List of extramural grants awarded from 7/1/2014-6/30-2015

The LB595 Molecular and Cellular Mechanisms of Smoking-Related Lung Disease Program: *Targeting Protein Kinase G (PKG) to Treat Early-Life ECS Exposure Related Lung Disease*

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

DEVELOPMENT PROGRAM PROGRESS REPORT
Thomas F. Murray, PhD, Principal Investigator

The Creighton University Cancer and Smoking Disease Research Program announced the opportunity for faculty to apply for Development Grants. There was no award competition during the fiscal year 2013-2014 due to the end of the five-year funding cycle.

The following investigators have completed the second year of their Development projects and requested no-cost extensions for an additional year:

PI: Patrick Swanson, PhD, Department of Microbiology and Immunology
Title: Role of Prolactin 2a1 in B Cell Development and Leukemogenesis

PI: Amy Arouni, MD, Department of Medicine/Cardiology
Title: Characteristics and Use Patterns of New e-Cigarette Users

The following investigator has completed her Development project:

PI: Laura Hansen, PhD, Department of Biomedical Sciences
Title: Oral Carcinogenesis and ADAM12

The following investigator has completed his start-up Development project:

PI: Peter Oldenburg, PhD, Department of Pharmacology
Title: Airway Responsiveness and Inflammation is Altered by Tobacco Smoke Extract in Allergic Mice

A new competition for Development projects was held in the spring of 2015.

Full reports follow this page.

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

DEVELOPMENT PROGRAM PROGRESS REPORT
Thomas F. Murray, PhD, Principal Investigator

Role of Prolactin 2a1 in B Cell Development and Leukemogenesis
Principal Investigator: Patrick Swanson, PhD

I. Progress Report Summary

A. Specific Aims

The original specific aims are as follows; they have not been modified:

1. Compare Prl2a1 expression levels in transgenic mice prone to indolent or aggressive $CD5^+$ B cell lymphocytosis, and identify Prl2a1-binding cells in these animals.
2. Determine levels of constitutive and Prl2a1-inducible Jak2/Stat5 signaling, Stat5 processing, and proliferation in transgenic mice prone to indolent or aggressive $CD5^+$ B cell lymphocytosis.
3. Establish the requirement for Prl2a1 expression for normal B cell development and $CD5^+$ B cell accumulation in transgenic mice prone to $CD5^+$ B cell lymphocytosis.

B. Studies and Results

Specific Aim 1:

In the previous progress report, we described the successful FACS purification of normal splenic follicular B cells ($CD19^+CD5^-$) from wild-type mice and splenic $CD19^+CD5^+$ B cells from dnRAG1, Eμ-TCL1, or DTG mice that develop a $CD5^+$ lymphocytosis resembling monoclonal B cell lymphocytosis (dnRAG1 mice), or indolent or aggressive chronic lymphocytic leukemia (Eμ-TCL1 mice or DTG mice, respectively) and detection of Prl2a1 in culture supernatants of $CD5^+$ B cells from dnRAG1 and DTG mice by immunoblotting. We have reproduced and extended that preliminary data to include peritoneal $CD5^+$ B cells from wild-type mice, as shown in Fig. 1A. As noted previously, the size of the detected band is consistent with the secreted polypeptide that lacks the signal peptide. Consistent with this hypothesis, we show here that treating cultured dnRAG1 splenocytes with monensin results in lower levels of Prl2a1 in culture supernatants and a corresponding increase in Prl2a1 levels in cells recovered after culture (Fig. 1B).

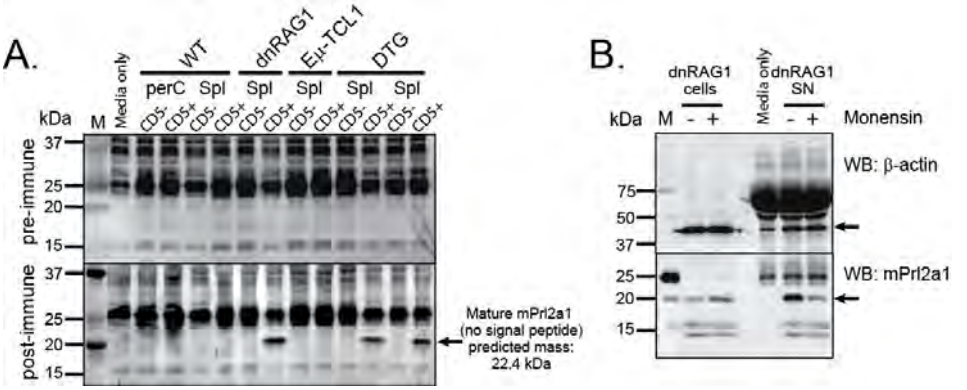


Figure 1. $CD5^+$ B cells from dnRAG1 and DTG mice secrete Prl2a1. (A) Culture supernatants from FACS-isolated $CD5^-$ or $CD5^+$ B cells from the peritoneal cavity (perC) or spleen (Spl) of WT, dnRAG1, Eμ-TCL1, or DTG mice were collected at 72h and analyzed by immunoblotting using pre- or post-immune serum from rabbit 8860. (B) Splenocytes from dnRAG1 mice were cultured in the absence or presence of monensin for 24 h. Cells and culture supernatants were collected and analyzed by Western blotting (WB) using antibodies specific for β-actin (top panel) or mPrl2a1 (bottom panel).

As described in the previous progress report, we were unsuccessful in detecting Prl2a1-binding cells by flow cytometry using partially purified His6-mPrl2a1-myc (which was used to screen Prl2a1-specific anti-serum). As described under Aim 2, we have successfully expressed and purified large quantities of untagged forms of mature murine prolactin (mPrl) and mPrl2a1. We have successfully conjugated these proteins with an Alexa647 dye at a level of 1-2 molecules per protein

(data not shown), but these proteins failed to specifically stain cells in a manner that was sensitive to competitive inhibition by unlabeled protein. To address this issue, we are generating human secreted alkaline phosphatase fusion proteins with Prl and Prl2a1 to identify Prl2a1-binding cells using the approach described by the Soares group.

Specific Aim 2:

As described in the previous progress report, we successfully cloned and purified mature mPrl and mPrl2a1 from bacterial inclusion bodies using denaturation/renaturation procedures and chromatographic approaches similar to those reported by others to purify recombinant prolactin. However, we described difficulties purifying large quantities of these proteins using the BL21(DE3)pLysS system, so we changed to a rhamnose inducible system using the *E. coli* strain KRX. We are now able to purify milligram quantities of these proteins suitable for structural analysis and bioassay.

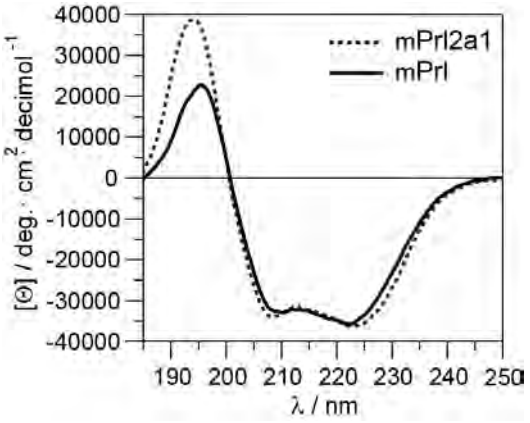


Figure 2. Purified mPrl and mPrl2a1 are structurally similar. Background-corrected ECD spectra were obtained for mPrl (black line) and mPrl2a1 (dashed line) in 10 mM potassium phosphate buffer (50 mM NaCl, pH 8.0) at 20°C.

In collaboration with Dr. Sandor Lovas, we compared the electronic circular dichroism (ECD) spectra of purified mPrl and mPrl2a1 (Fig. 2). Both spectra indicate the presence of a negative band at 208 nm corresponding to an $n\pi^*$ transition from static field mixing, as well as exciton splitting of a $\pi\pi^*$ absorption band into two components around 195 nm and 208 nm that are consistent with the presence of α-helicity in both proteins. The similarity of both spectra suggests that mPrl and mPrl2a1 are structurally quite comparable.

We also compared the activity of the purified prolactin proteins using the well-established rat Nb2 lymphoma cell bioassay for prolactin. In this bioassay, purified mPrl2a1 alone failed to stimulate Nb2 cell proliferation at concentrations of up to 1000 ng/mL. By contrast, purified mPrl was found to stimulate Nb2 cell proliferation in a dose-dependent manner, with an ED_{50} of ~0.1 ng/mL (Fig. 3). A similar level of stimulation was detected using commercial rat Prl as a positive control. Importantly, addition of excess mPrl2a1 (up to 100,000-fold) had no effect on mPrl-dependent Nb2 cell proliferation at any concentration of mPrl tested in this assay, nor did it inhibit Nb2 cell growth at various concentrations of maintenance media. Thus, we conclude that mPrl2a1 neither stimulates nor antagonizes Prl-dependent cell proliferation.

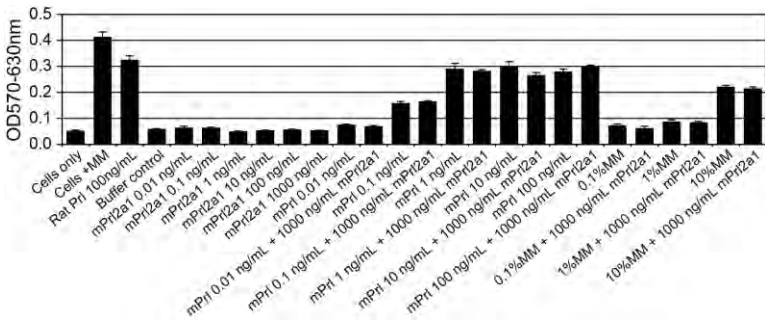


Figure 3. mPrl2a1 neither stimulates nor antagonizes Prl-dependent proliferation of Nb2 cells. Nb2 cells were cultured in triplicate in maintenance media (MM) or media containing horse serum and treated with rat Prl, or purified mPrl and/or mPrl2a1 at various concentrations. Cells were harvested 72 h later and analyzed using an MTT assay.

Specific Aim 3:

This aim involved generating Prl2a1-knockout mice. As described in the previous progress report, the Prl2a1$^{tm1e(KOMP)Wtsi}$ targeted ES cell lines available through the KOMP repository all failed their quality assurance testing, and attempts to generate new ES cell lines using an available conditional targeting vector for Prl2a1 were unsuccessful. As an alternative, we pursued a CRISPR/Cas9 approach to generate a conventional Prl2a1 gene knockout using the UC Davis Mouse Biology Program. Targeting exon 2, they have generated one founder with a confirmed indel resulting in a frameshift. This founder animal is currently being bred to generate offspring to establish our breeding colony.

C. Significance

The findings in Aims 1 and 2 are significant because they represent the first evidence of a prolactin-like protein being expressed *and secreted* from B cells and the first report of the purification, structural characterization, and bioactivity analysis of mPrl2a1. Aim 3 is significant because this represents the first reported knockout of Prl2a1 in mice. If we are able to establish the cells and receptors targeted by Prl2a1, and demonstrate that Prl2a1 functions as an immunomodulatory factor, this would be an important breakthrough in understanding the roles of this hormone.

II. List of refereed publications germane to this project from 7/1/2014–6/30/2015

None yet, but a manuscript containing the data presented here was submitted to *Molecular and Cellular Endocrinology* on July 2, 2015.

III. List of extramural grants submitted from 7/1/2014–6/30/2015

Agency: NIH/NIAID R21 AI119829-01, submitted October 2014.
Role: PI
Title: Implications of B10-Like Cell Expansion in a Model of Impaired Receptor Editing
Dates: 07/01/2015 to 06/30/2017
Amount: $400,125 total

Agency: NIH/NIGMS 3R01GM102487-03S1
Role: PI
Title: A Flow cytometer for Multi-Color Cell Analysis in a Multi-User Facility
Dates: July 1, 2014 to June 30, 2015
Amount: $100,950

Agency: NIH/OD 1S10 OD021614-01
Role: PI
Title: FACSAria Fusion Flow Cytometer
Dates: February 1, 2016 to January 31, 2017
Amount: $701,190

IV. List of extramural grants awarded from 7/1/2014–6/30/2015

Agency: NIH/NIGMS 5R01GM102487-04
Role: PI
Title: Role of VprBP in B Cell Development and V(D)J Recombination
Dates: July 1, 2015 to June 30, 2016
Amount: $276,436

Agency: NIH/NIGMS 3R01GM102487-03S1
Role: PI
Title: A Flow Cytometer for Multi-Color Cell Analysis in a Multi-User Facility
Dates: July 1, 2014 to June 30, 2015
Amount: $100,950

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

DEVELOPMENT PROGRAM PROGRESS REPORT
Thomas F. Murray, PhD, Principal Investigator

Characteristics and Use Patterns of New e-Cigarette Users
Principal Investigator: Amy Arouni, MD

Characteristics and Use Patterns of New e-Cigarette Users (primary study):
We have enrolled 46 participants into this study; the majority of whom are female (67.4%) and white (91.3%). The age of the participants ranged from 21 to 70 years, with a mean age of 40.2 years. More than half of study volunteers have private health insurance (53%), while the others are nearly evenly split between having Medicare and/or Medicaid (24%) or being uninsured (27%).

Baseline cigarette smoking patterns of those enrolled are detailed below:

Age at smoking initiation, mean (range)		17 (9-63)
Number of years smoking, mean (range)		22 (3-25)
# Cigarettes smoked per day, n (%)		
	0-10	12 (27)
	11-20	20 (44)
	21-30	8 (18)
	31+	5 (11)
Fagerstrom score, median (range)		5 (0-10)
Reason for purchasing an e-cigarette, n (%)		
	Quit smoking, but keep nicotine	11 (24)
	Quit smoking and quit nicotine	25 (56)
	Have nicotine where smoking is not allowed	5 (11)
	Other	4 (9)

The attrition rate in this study has been high, with 35 participants reporting at month 1 (a 23.91% loss from baseline), 27 participants reporting at month 3 (a 41.30% loss from baseline), and 19 reporting at month 6 (a 58.70% loss from baseline). Two participants withdrew from the study due to health changes following e-cigarette initiation (chronic sore throat and nausea), and two others wished to withdraw because they were no longer using their device and did not want to continue follow-up visits. We suspect the remaining people who were lost to follow-up discontinued participation primarily due to discontinuation of e-cigarette use; however, we have been unable to confirm this despite multiple contact attempts.

Smoking habits: The average study volunteer smoked around one pack per day at the start of the study; however, the daily cigarette count dropped dramatically as time progressed in those continuing follow-up. Average cigarettes smoked per day were 19.74, 6.98, 7.41, and 5.18 at baseline, month 1, month 3, and month 6, respectively. Non-daily smoking was reported by 10 (of 35) participants at month 3, and by 17 (of 19) participants at month 6. However, no participants ceased using both traditional cigarettes *and* e-cigarettes during the 6-month follow-up period.

Nicotine Intake: One of the many concerns of e-cigarette use is nicotine intake, particularly the possibility of nicotine toxicity. We monitored nicotine intake, assessed by salivary cotinine, at each study visit to assess our participants' management of nicotine during their study follow-up period. Our preliminary data analysis is limited in that it only includes three of the four study visits (baseline, 1 month, and 3 months), and only a portion of those completing follow-up through the 3-month follow-up visit (n=32). We found that participants' nicotine intake did not significantly change from baseline despite a reduction in cigarettes per day. Therefore, regardless of whether a person was exclusively smoking cigarettes, using both cigarettes (at a reduced rate) and an e-cigarette, or only using an e-cigarette (vaping), the level of nicotine the person received remained fairly consistent.

Abstract Submissions: We submitted two abstracts describing our lung function and nicotine intake results (see below). Both abstracts were accepted for presentation at the Society for Research on Nicotine and Tobacco Annual Meeting in February 2015. *Changes in Lung Function over Time after Initiation of e-Cigarette Use* was presented as an oral session. It includes data on all participants through the entire 6-month follow-up period, and highlights the encouraging results we found in participants' forced expiratory volume (FEV1) and forced vital capacity (FVC) measurements. *Salivary Cotinine Levels over the First Three Months of e-Cigarette Use* presents preliminary data on participants' ability to self-titrate nicotine intake while experimenting with e-cigarettes.

Changes in Lung Function over Time after Initiation of e-Cigarette Use

Shavonne Washington-Krauth MA, Tammy Burns PharmD, Ryan Walter MS, Amy Arouni MD; Creighton University School of Medicine, Omaha, Nebraska

Background: Electronic cigarettes (e-cigarettes) are relatively new in the global tobacco market, with the first e-cigarette introduced in 2003. A paucity of literature exists regarding the short- and long-term health effects of e-cigarettes. The current study uses data from new e-cigarette users to model how lung function changes over time.

Methods: This observational study enrolled 46 participants within the 7 days following their first purchase of an e-cigarette. Visits occurred at baseline, 1, 3, and 6 months after purchase. Lung function was a primary outcome measured by handheld spirometry using percent of predicted Forced Expiratory Volume in 1 second (FEV1%) and percent of predicted Forced Vital Capacity (FVC%). Because no smoking restrictions were placed participants, we adjusted for participants' self-reported average cigarettes smoked per day at each visit. Linear mixed-effects models were employed for each outcome separately—polynomial effects were evaluated and person-mean centering was used to partition the between- and within-participant effects of the time-varying cigarettes smoked predictor.

Results: The attrition rate was high, with 35 participants reporting at month 1, a 23.91% loss from baseline, and 27 participants reporting at month 3, a 41.30% loss from baseline, and 19 reporting at month 6, a 58.70% loss from baseline. Non-daily smoking was reported by 10 participants at month 3. Average cigarettes smoked per day were 19.74, 6.98, 7.41, and 5.18 at baseline, month 1, month 3, and month 6, respectively. Non-daily cigarette smoking was reported by 17 participants at month 6; however, no participants ceased using both smoking and e-cigarettes.

For FEV1% predicted, approximately 68.29% of the variability was a result of between-participant differences, where 95% of participants were expected to have

FEV1% predicted between 63.21% and 117.16%. A significant interaction effect was observed between linear change in FEV1% across visits and the participant's average number of cigarettes per day across visits. More specifically, when participants smoked their usual number of cigarettes per day across all visits, FEV1% increased non-significantly by 0.49% across visit (p=0.31, 95% CI=-0.46 to 1.44; see Figure 1, red line). However, a one-cigarette per day decrease below the participant's usual number of cigarettes smoked per day resulted in the linear effect of FEV1% increasing significantly by an additional 0.18% per visit (95% CI=0.04 to 0.32, p<.05). Thus, a significant increase in FEV1% across visits was observed for individuals who decreased their usual level of smoking at a given visit by as little as three cigarettes (see Figure 1; blue line).

For FVC% predicted, approximately 85.35% of the variability was a result of between-participant differences, where 95% of participants were expected to have FVC% predicted values between 63.51% and 118.31% at baseline (see Figure 1; thin lines). A significant fixed quadratic effect was observed with significant average linear increase in FVC% across visits of 2.95% being mitigated significantly by an average of 0.69% per visit (both linear and quadratic effect p<.05; see Figure 2, thick black line). Note that change in predicted FVC% across visits plateaued by month 6 (i.e., predicted change specifically at month 6=-1.19, p=0.17).

Conclusion: Lung function, assessed by spirometry, improved significantly from baseline despite the majority of users continuing to smoke cigarettes. E-cigarettes may result in an improvement in lung function with long-term use and reduction of cigarette intake. The safety of long-term use of e-cigarettes has yet to be established. We will continue to collect data on participants up to one year from enrollment.

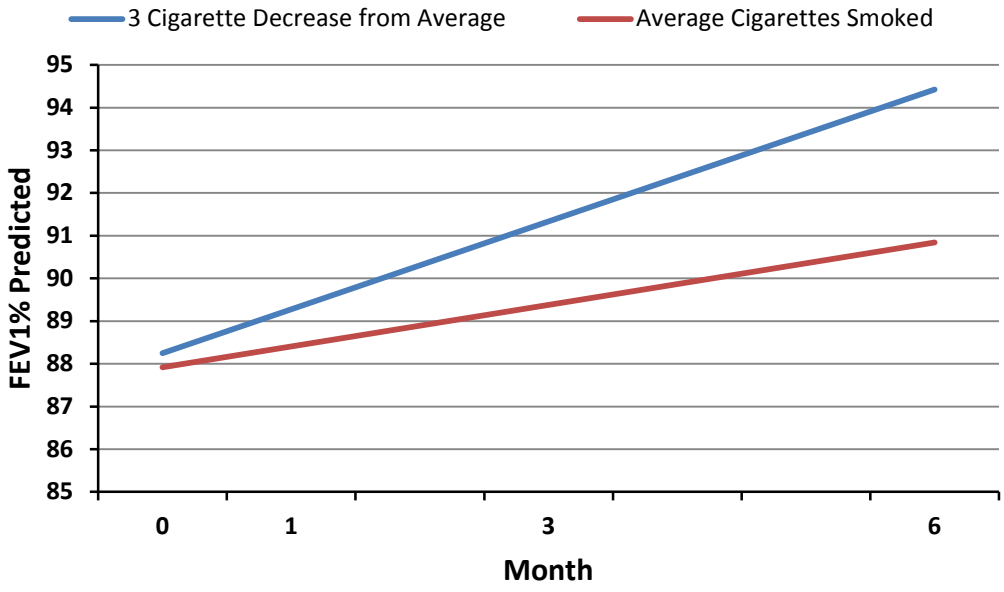


Figure 1. FEV1% predicted interaction effect between linear change and a participant's average (aka, usual) level of smoking.

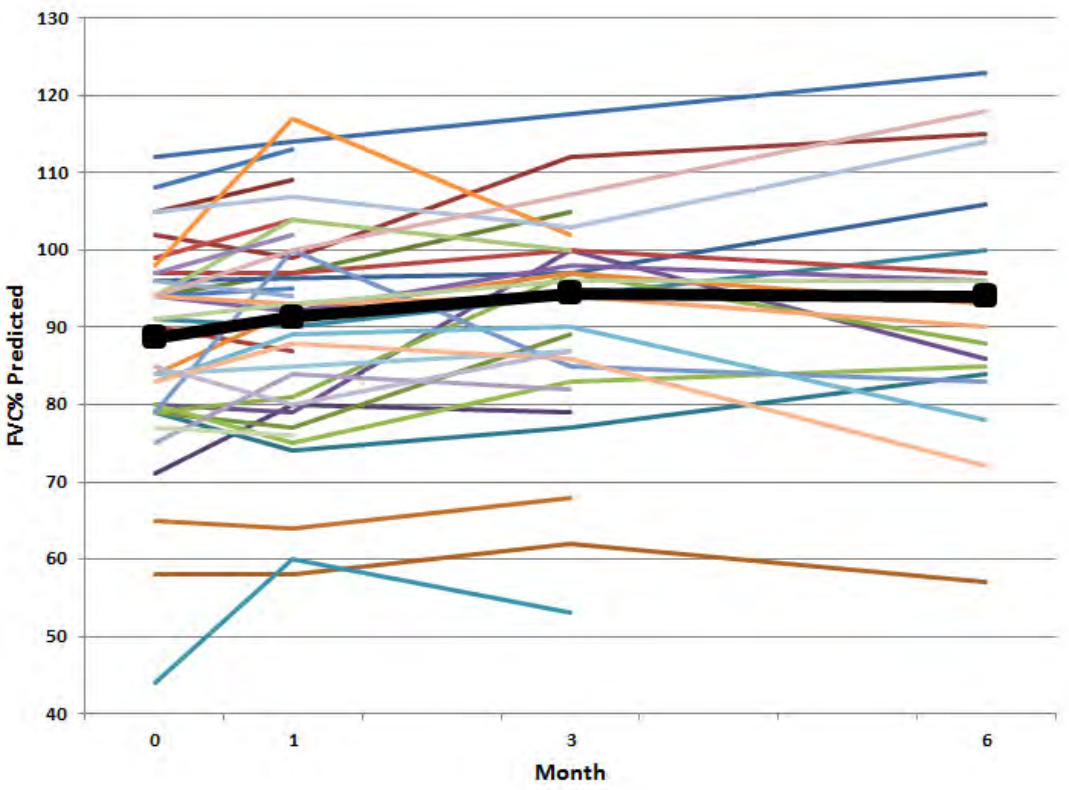


Figure 2. FVC% predicted showing quadratic change over months.

Salivary Cotinine Levels over the First Three Months of e-Cigarette Use
Tammy Burns PharmD, Shavonne Washington-Krauth MA, Ryan Walters MS, Amy Arouni MD; Creighton University School of Medicine, Omaha, Nebraska

Background: Electronic cigarettes (e-cigarettes) are relatively new in the global tobacco market, with the first e-cigarette introduced in 2003. A paucity of literature exists regarding the use patterns and impacts on other tobacco use after initiation of e-cigarette use. The current study uses data from new e-cigarette users to model how salivary cotinine levels change over time.
Methods: This observational study enrolled 41 participants within seven days of their first e-cigarette purchase. Visits occurred in our research clinic at baseline, 1 month, and 3 months after purchase. Salivary cotinine, measured by ELISA/EIA (Salimetrics, Inc., Carlsbad, CA), served as the primary outcome to assess changes in nicotine intake. Because no smoking restrictions were placed on participants, models were adjusted for participants' self-reported average cigarettes smoked per day measured at each visit. Linear mixed-effects models were employed—polynomial effects were evaluated and person-mean centering was used to partition the between- and within-participant effects of the time-varying cigarettes smoked predictor.
Results: Non-daily smoking was reported by 10 participants at month 3. One participant was using nicotine-free liquid in the e-cigarette, but no participants abstained from both smoking and e-cigarette use. Results indicated no change in salivary cotinine over time; however, a between-participant effect of cigarettes was observed with a one-cigarette per day average increase compared to other participants, resulting in cotinine levels increasing by an average of 17.14 ng per ml.

Mean cigarettes per day were 19.7, 9.8, and 10.6 at baseline, 1 month, and 3 months, respectively.
Conclusion: Nicotine intake, assessed by salivary cotinine, did not significantly change from baseline despite a reduction in cigarettes per day. E-cigarettes may lead to self-titration of nicotine intake to maintain a similar intake as smoking. We are continuing to collect data from participants up to one year from enrollment.

Future Plans: We hope to enroll more participants into this study to increase our numbers and thereby strengthen our results. Recruitment is more difficult now that ENDS are no longer as novel as they were when first initiating this study, so we plan to expand our recruitment efforts into additional "vape shops" in the area. This should help us capture more new users within the small 7-day use initiation window required for study enrollment.

We are also beginning to look at the remaining cotinine sample results recently received. This analysis will increase the number of participants with 3-month cotinine results and provide the first look at 6-month levels. Therefore, it is possible the results of the preliminary cotinine analysis will change once all participants' data points are included.

In addition to reanalyzing the cotinine data, we plan to analyze additional data collected throughout the study. Additional data collected include: carbon monoxide, blood pressure and heart rate, device satisfaction, use patterns identified through smoking/vaping diary tracking, and smoking self-efficacy. Finally, we plan to begin writing manuscripts for submission to peer-reviewed journals and continue seeking outside funding opportunities to further support our work in this area.

Carbon Monoxide Levels and Changes in Acute Pulmonary Function Following e-Cigarette Use (sub-study):
We noticed during the early stages of our e-cigarette observation study that some participants reporting no or non-daily cigarette smoking had higher carbon monoxide (CO) measurements than participants in our previous smoking cessation studies. Although the levels were rarely over those of a person who doesn't smoke (7 ppm maximum), they were not the typical 2-4ppm seen in those who quit smoking using either traditional FDA-approved medications or "cold turkey." This made us wonder whether these elevated levels were random, due to an unrelated factor, or possibly due to particular ENDS used or even the e-juice being vaped. This led to the addition of a CO and lung function sub-study for exclusive ENDS users.

We are enrolling exclusive ENDS users (defined as daily use of an e-cigarette for at least one month without any other tobacco use) and comparing the CO and acute lung function changes (FEV1 and FVC) measured when vaping from one of two devices filled with one of three flavors of e-juice containing either 0 or 16mg of nicotine. Currently, we have screened 26 people and enrolled 14 participants, so no preliminary results are available. Like the primary observational study, most participants are female (n=9) and white (n=13). Participant ages range between 34 and 66 years.

Future plans: We plan to continue enrolling participants into this study to reach our goal of 100 people. Data analysis will compare results between devices, juices, and the various device/juice combinations. Results will be submitted for presentation at relevant scientific meetings and submission to peer-reviewed journals.

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

DEVELOPMENT PROGRAM PROGRESS REPORT
Thomas F. Murray, PhD, Principal Investigator

Oral Carcinogenesis and ADAM12
Principal Investigator: Laura Hansen, PhD

I. Progress Report Summary

A. Specific Aims

Aim 1B, to evaluate ADAM12 and EGFR/HER2 receptor expression during oral carcinogenesis, has been somewhat modified as we determined in the past year whether an alternative *Adam12* transcript exists in mouse oral and other tissues.

B. Studies and Results

Our previously published work (2) suggested the protease ADAM12 (a disintegrin and metalloproteinase 12) might cooperate with the EGFR receptor tyrosine kinase in human head and neck squamous cell carcinoma (HNSCC). Consequently, using genetically-engineered *Adam12* null and wild type control mice, we tested the hypothesis that genetic deletion of *Adam12* reduces the development and progression of oral cancers in a mouse chemical carcinogenesis model. However, inconsistent qPCR results for *Adam12* knockout led us to investigate possible alternative transcripts that may be expressed in the *Adam12* mutants. We also wanted to check the integrity of the knockout, in case mutation or breeding mistakes had made it ineffective. The knockout was constructed by replacing the first exon of ADAM12 with a neomycin cassette (1). The NCBI database contains an *Adam12* transcript with an alternative first exon (Fig. 1). We designed primers for RT-PCR and genomic PCR assays to address this issue (Fig. 2 and Table 1).

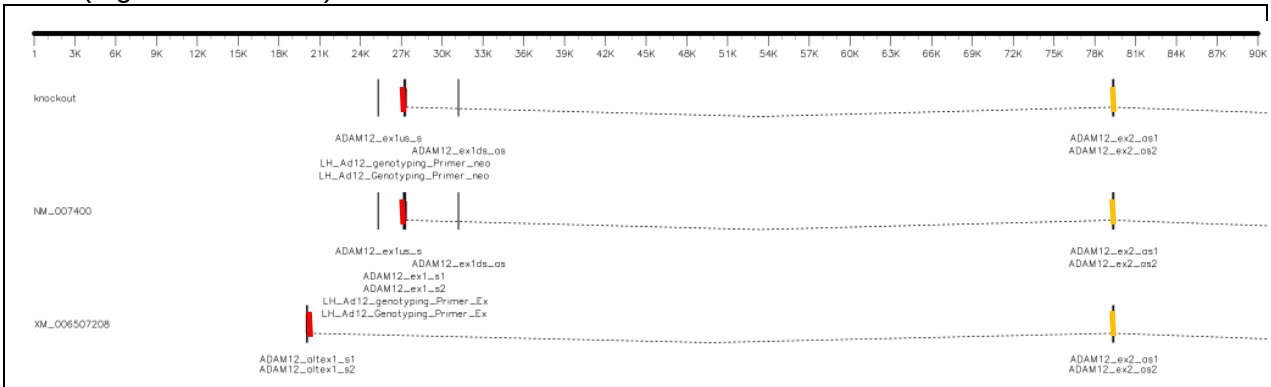


Figure 1. Splice variants of mouse *Adam12* in genomic context with genotyping primer locations. Exons/introns for Adam12 knockout allele (top), exons for wild type primary transcript (middle), and exons of putative alternate transcript (bottom). Gray vertical lines indicate primer locations, with primer name underneath. Red lines indicate exon 1 and orange lines indicate exon 2.

Nested PCR primers on exon 2 were paired with nested PCR primers from exon 1 and the

alternative exon 1 for RT-PCR (Fig. 2). From 8 pups, two yielded strong bands (Fig. 3). These bands were about 100bp shorter than predicted from the database entry. When sequenced, we found that a truncated alternate exon spliced onto exon 2 (not shown).

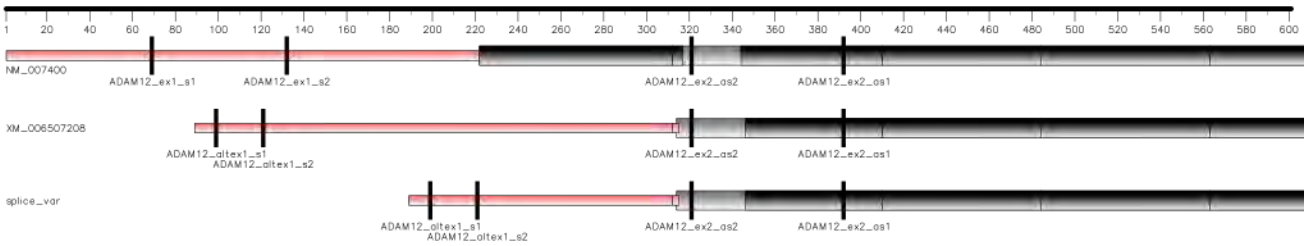


Figure 2. Splice variants of *Adam12* transcripts with nested RT-PCR primer locations. Primers are indicated by vertical black bars. Red: UTR.

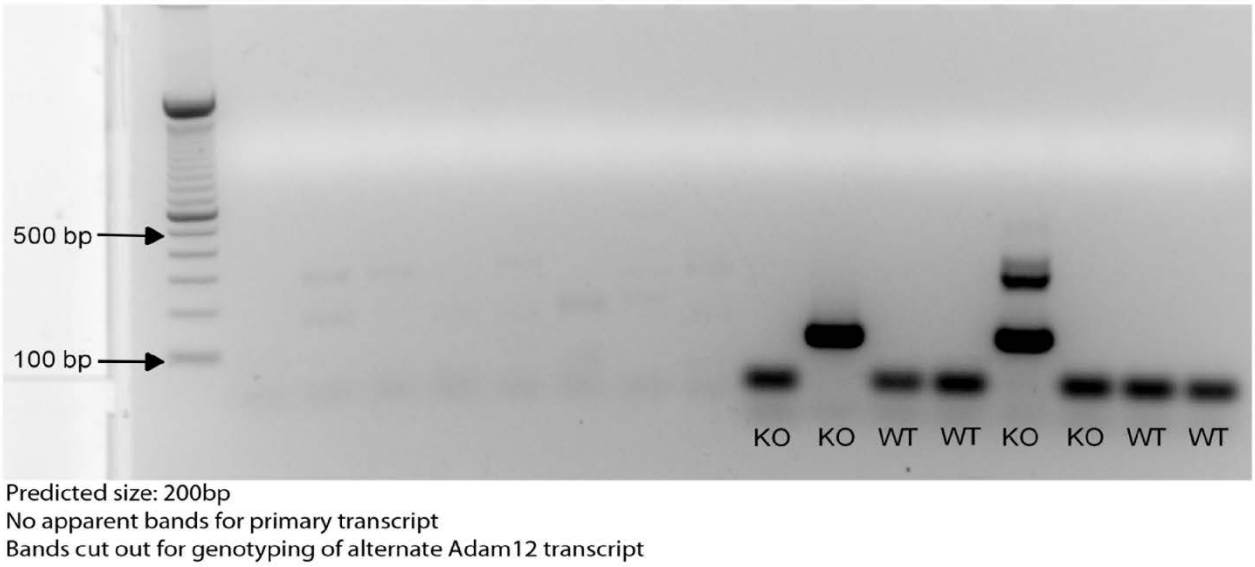


Figure 3. Nested RT-PCR of *Adam12* splice variants in *Adam12* wild type and mutant mice. Each lane represents RNA from a separate mouse.

We then wanted to confirm expression of this transcript with qPCR and compare its levels with any of the primary transcript that may be expressed. The small difference between the novel splice variant and the alternate transcript from NCBI means that these primers may produce a band for each.

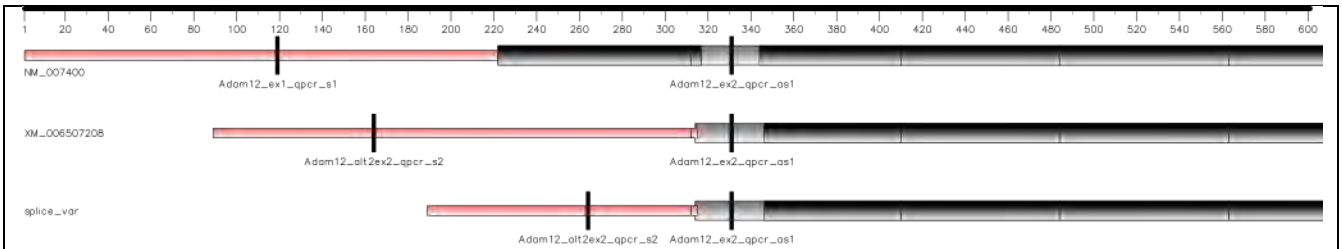


Figure 4. Locations of real-time RT-PCR primers on native and alternatively spliced Adam12.

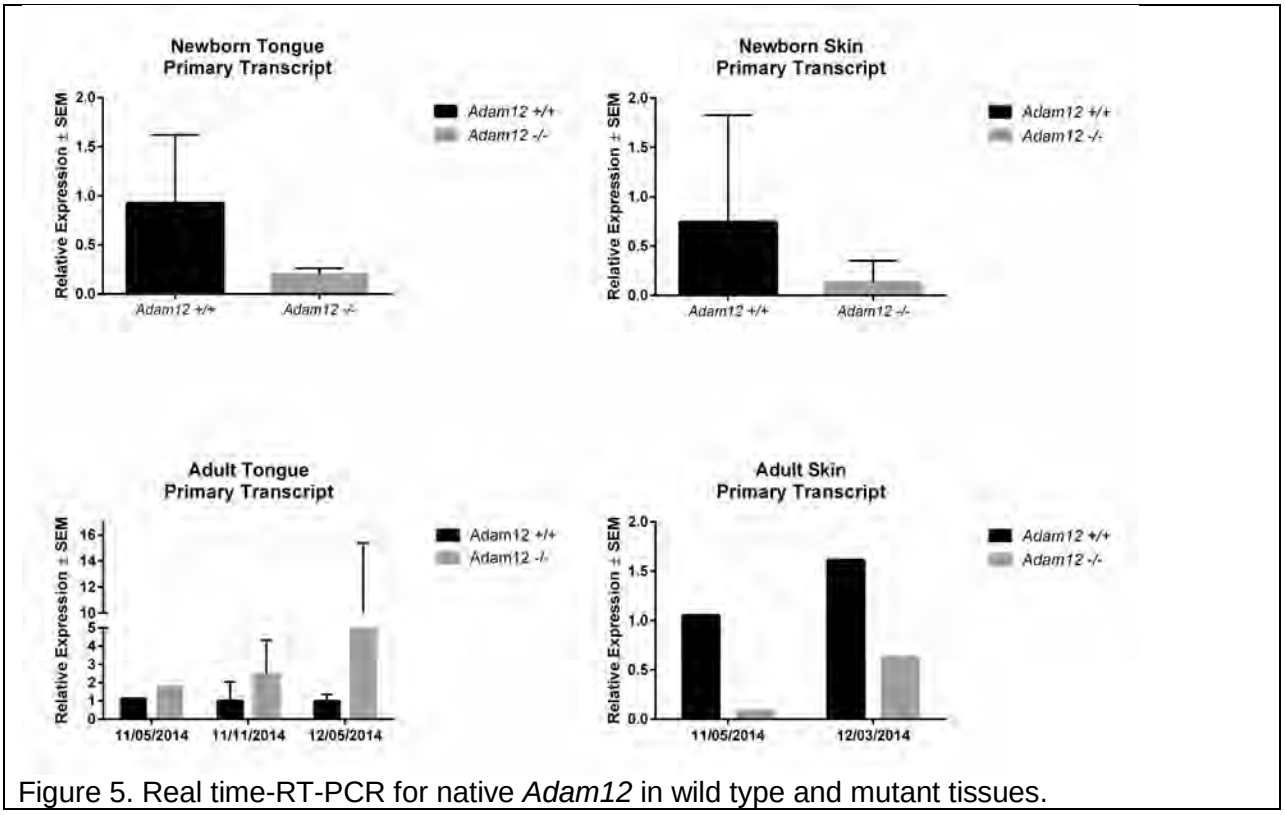


Figure 5. Real time-RT-PCR for native *Adam12* in wild type and mutant tissues.

This qPCR using primers shown in Fig. 4 confirmed reduced expression of the primary transcript in knockout skin in both adult and pup skin (Fig. 5, right hand panels). Samples from tongue were inconsistent (Fig. 5 left hand panels). The splice variant qPCR performed poorly on all samples (Fig. 6). The melt curves for both of these assays were not good.

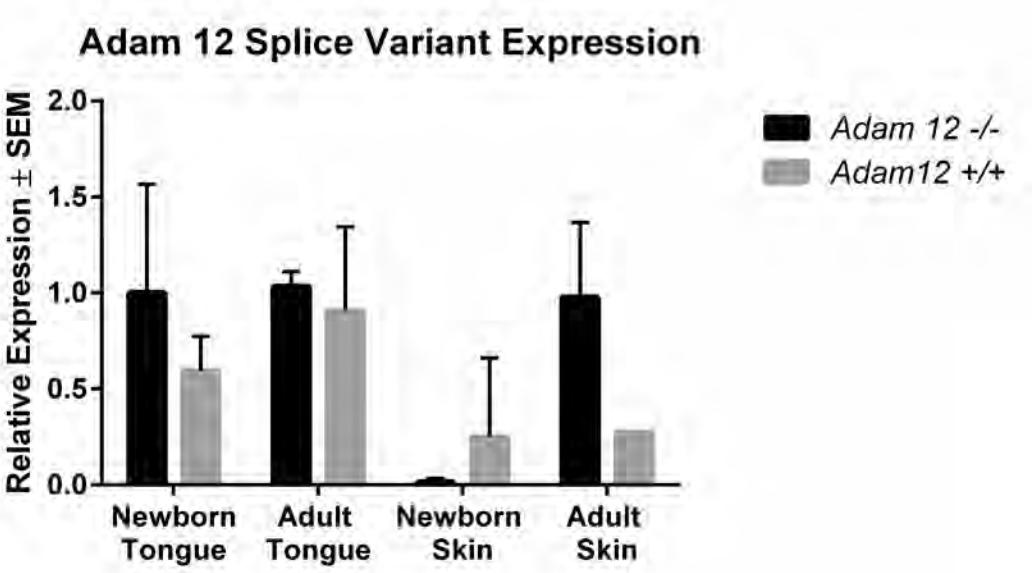


Figure 6. qPCR for splice variants of Adam12 in wild type and mutant tissues.

Additionally, we examined the genomic structure in this region. Genotyping primers that align to NEO were positive in homozygous knockouts while primers that aligned to the primary exon 1 failed to produce a band (Fig. 7). This indicates that genotyping had been done correctly and the knockout contained a NEO sequence and a disrupted primary exon 1.

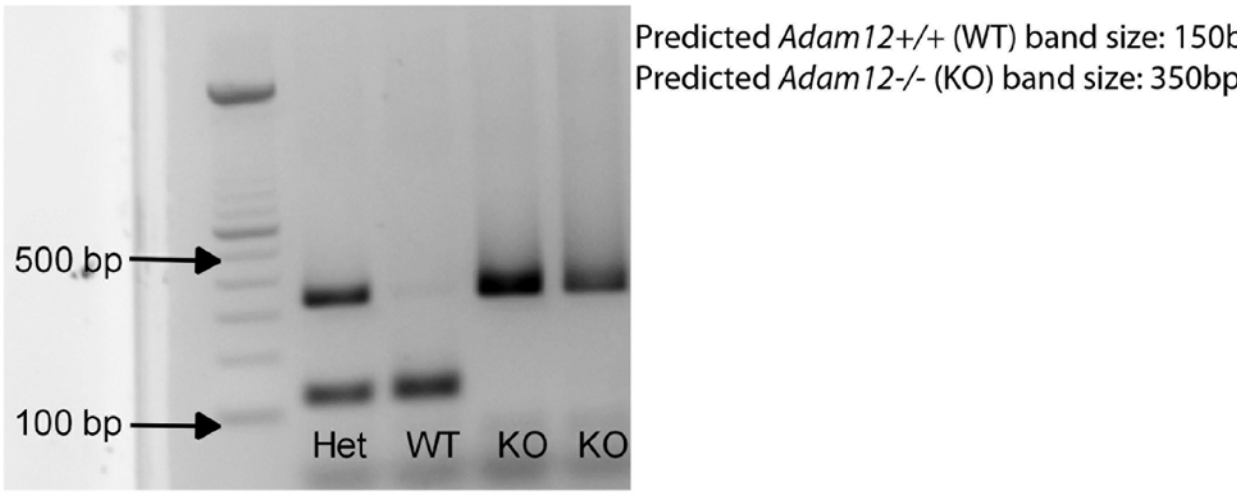


Figure 7. Genotyping PCR showing wild type and mutant bands. Each lane represents DNA from a separate mouse.

We also attempted to amplify a band that contains the NEO cassette on one side and adjacent genomic regions on the other. Disagreement between the NCBI sequence and the diagram in the paper documenting the knockout (1) made it hard to design appropriate genomic primers. Placing the primers near known restriction sites that exist in both required amplification of 2-3kb. This was attempted several times with elongation times of 3 minutes and high quality column purified gDNA, but we were unable to produce a band (not shown).

Table. 1. PCR primers.

Adam12-altex1-qpcr-s1	CATTGCCTTTCAGACTCCA
Adam12-altex1-qpcr-s2	TGCCTTTCAGACTCCAGACA
Adam12-ex2-qpcr-as2	CCTCTCTGGTCCCACAAACT
Adam12-ex2-qpcr-as1	GCCACTTCGTAAGTTCCTCTCT
Adam12-alt2ex2-qpcr-s2	GCTACCGACCCTGGTCACA
Adam12-alt2ex2-qpcr-s1	GCCACTTCGTAAGTTCCTCTCT
Adam12-ex1-qpcr-s1	GCAGACTCAGGGCAGTAGGA
Adam12-ex1-qpcr-s2	GGCAGACTCAGGGCAGTAG
ADAM12-ex1us-s	GTAGGCACATTCCGGTGAGT
ADAM12-ex1ds-as	TGCACACCGTATATGCCCTA
ADAM12-ex1-s1	AAGGCTAGACTCGCTGCTCA
ADAM12-ex1-s2	AGTAGGACTTCCCCCAGCTC
ADAM12-ex2-as1	TTGGCTGGGATGCTCTGT
ADAM12-ex2-as2	GTTCCTCTCTGGTCCCACAA
ADAM12-altex1-s1	CCAGAGGCCTGTTCTGAGAC
ADAM12-altex1-s2	GCTAAAGGGCCACCTTGTG
LH 15 Adam12 F (MS)	CAGGTGGAATGCTCTTCTCA
LH 16 ADAM12 R (MS)	CTTTGGGTGCTTTGTCCAAT
LH Ad12 genotyping Primer Neo	TGGAGAGGCTATTCGGCTATGACTGGG
LH Ad12 genotyping Primer Neo	ATGCAGCCGCCGCATTGCAT
LH Ad12 genotyping Primer Ex	GCGCTCTGCCATTGTCGCCG
LH Ad12 Genotyping Primer Ex	GGCAGACTCAGGGCAGTAGGACTTCCC

C. Significance

Our analyses of Adam12 expression in this previously published mutant mouse has not revealed a consistent loss of *Adam12* expression in the mutant mouse tissues, while analysis of the *Adam12* gene reveals a potential alternative start site that could provide for some Adam12 expression in the knockout mouse. The data produced do not fully explain the results obtained with the Adam12 mutant tissues, but do suggest that an alternate *Adam12* transcript may exist that could impact analysis of Adam12 function in these animals.

II. List of refereed publications germane to this project from 7/1/2014–6/30/2015

1. Rao, V.H., Vogel, K., Yanagida, J.K., Marwaha, N., Kandel, A., Trempus, C., Repertinger, S.K., and **Hansen, L.A**. Erbb2 up-regulation of ADAM12 expression accelerates skin cancer progression. In press in *Molecular Carcinogenesis*, 2015

2. Lehman, J. Association of ADAM12 with head and neck cancer development. Master's Thesis, 2015.

III. List of extramural grants submitted from 7/1/2014–6/30/2015

INBRE PI: M. Nichols (CoI: Hansen)
Dates: 5/1/15-4/30/16
Title: Metabolic imaging of disease progression in skin cancer by FLIM
Award: $92,300 (total direct)

CURAS Project PI: Hansen
Title: Cytoplasmic CDC25A localization and suppression of apoptosis in cancer cells.
Dates: 7/1/15-6/30/16
Award: $5,000

ProTransit Nanotherapy Project PI: Hansen
Title: The Protective Efficacy of PLGA Nanoparticles Encapsulating Antioxidant Enzymes against UV Radiation Exposure
Dates: 7/1/14-12/30/15
Award: $5868

LB506 PI: Hansen
Title: Estrogen receptor alpha and squamous cell carcinoma of the skin
Proposed Dates: 7/1/2015-6/30/2016
Proposed Award: $50,000

NIH R01 PI: Hansen
Title: Mechanisms of UV-induced skin carcinogenesis (Competitive Renewal)
Proposed Dates: 2015-2020
Proposed Award: $1,838,406

NIH STTR PI: Madsen (Multi-PI Hansen)
Title: Developing Pro-NP for skin cancer prevention
Proposed Dates: 9/1/15-8/31/16
Proposed Award: CU subcontract $97,523

IV. List of extramural grants awarded from 7/1/2014–6/30/2015

CURAS Project PI: Hansen
Title: Cytoplasmic CDC25A localization and suppression of apoptosis in cancer cells.
Dates: 7/1/15-6/30/16
Award: $5,000

ProTransit Nanotherapy Project PI: Hansen
Title: The Protective Efficacy of PLGA Nanoparticles Encapsulating Antioxidant Enzymes against UV Radiation Exposure
Dates: 7/1/14-12/30/15
Award: $5868

INBRE PI: M. Nichols (CoI: Hansen)
Dates: 5/1/15-4/30/16
Title: Metabolic imaging of disease progression in skin cancer by FLIM
Award: $92,300 (total direct)

Reference List

1. Kurisaki T, Masuda A, Sudo K, et al. Phenotypic analysis of Meltrin alpha (ADAM12)-deficient mice: involvement of Meltrin alpha in adipogenesis and myogenesis. Mol Cell Biol 2003; 23(1): 55-61

2. Rao VH, Kandel A, Lynch D, et al. A positive feedback loop between HER2 and ADAM12 in human head and neck cancer cells increases migration and invasion. Oncogene 2012; 31(23): 2888-98

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

DEVELOPMENT PROGRAM PROGRESS REPORT
Thomas F. Murray, PhD, Principal Investigator

Airway Responsiveness and Inflammation is Altered by Tobacco Smoke Extract in Allergic Mice
Principal Investigator: Peter Oldenburg, PhD

I. Progress Report Summary

This is the final report for this grant. Since the beginning, slow progress has been made and the anticipated proposed goals were not reached in a timely manner. The External Advisory Committee therefore recommended that this project be terminated to focus the research efforts of the laboratory on a separate NIH-funded project.

A. Specific Aims

The specific aims have not been modified. We hypothesized that cigarette smoke alters airway hyperresponsiveness (AHR) and airway inflammation in asthma.

- **Aim 1:** Characterize the effect of cigarette smoke extract on airway hyperresponsiveness.
- **Aim 2**. Characterize the effects of cigarette smoke extract exposure on lung inflammation in allergen-sensitized mice.
- **Aim 3.** Characterize *ex-vivo* cigarette smoke extract-induced changes in airway responsiveness.

B. Studies and Results

Aim 1 Progress: Progress was made in aim 3 to determine the optimum concentration of cigarette smoke extract (CSE) to be used to treat the mice to induce and measure altered airway hyperreactivity. We have determined that instilling 5% CSE would give optimum results. Equipment was purchased to allow us to measure changes in lung resistance and dynamic compliance in these mice. We have recently purchased a non-invasive airway mechanics (NAM) instrument to allow us to monitor changes in specific airway resistance (SRaw) in conscious unrestrained mice. This new system would have allowed us to track the progression of changes in airway resistance during the course of CSE treatment. This would have allowed us to answer the question of: What duration of treatment time was needed to see significant changes in airway reactivity?

Aim 2 Progress: We are able to sensitize mice to ovalbumin and observe increased pulmonary inflammation in the lungs of these mice. Based on previous studies using organic dust extract in mice, we anticipated seeing increased numbers of neutrophils present in the lungs of these mice following treatment with CSE. This would have contributed to the increased changes in airway reactivity that we would have observed in aim 1. Since the project was terminated, no further progress was made.

Aim 3 Progress: Progress was being made in this aim. We determined that the reason the airways in the precision-cut lung slices (PCLS) were not responding to methacholine

was due to the fact that the lungs were being overinflated with agarose by the new technician in the laboratory. Once this was remedied, we were able to treat PCLS with CSE and started to generate data. We concluded that 1% CSE had no effect on airway reactivity when compared to controls, 5% CSE increased the airway reactivity, and 10% CSE was toxic to the tissues. We observed an increase in airway reactivity to methacholine in PCLS following 1 and 24 hr CSE treatments using 5% CSE. To mimic allergic airways, TNF and CSE was added to the PCLS and an increase in airway reactivity to methacholine was observed, suggesting that the airways of allergen-sensitized mice would be more responsive to CSE. Since termination of the project, no further progress was made in this aim.

C. Significance

It is well described that smoking and cigarette smoke exposure results in increases in lung-related diseases. Despite the extensive research performed in mouse models of allergic asthma, the effects of cigarette smoke extract on allergen-induced airway responses in atopic asthmatic mice has not been extensively studied. The data generated from these aims would allow us to better understand how cigarette smoke affects the pulmonary inflammation and airway responsiveness of allergen-sensitized mice.

II. List of refereed publications germane to this project from 7/1/2014-6/30/2015.

None

III. List of extramural grants submitted from 7/1/2014-6/30/2015.

None

IV. List of extramural grants awarded from 7/1/2014-6/30-2015.

The LB595 Molecular and Cellular Mechanisms of Smoking-Related Lung Disease Program: *Targeting Protein Kinase G (PKG) to Treat Early-Life ECS Exposure Related Lung Disease*

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

DEVELOPMENT PROGRAM AWARDS
Thomas F. Murray, PhD, Principal Investigator

The Development Program assists faculty in developing pilot research projects related to cancer and smoking diseases. The goal of this program is to provide two years of support to investigators so they can develop fully realized projects meriting inclusion in one of the three Cancer and Smoking Disease Research Program Projects; in other cases, the project may develop into its own Research Program Project for a future inclusion in Creighton's Cancer and Smoking Disease Research Program. Investigators for the Development Pilot Projects are chosen through a competitive process that selects for funding the most promising and innovative research. Each year, a call for Pilot Projects is distributed for proposals. We received three applications for Developmental Pilot Projects, and all were reviewed by each External Advisory Committee member. The newly funded projects are as follows:

- "Regulation of ITIH3 by Nicotine and Tobacco Smoke through the CD44 Receptor" Deniz Yilmazer-Hanke, PhD, Department of Biomedical Sciences.

- "Acquired Resistance to Targeted Therapy in Mucinous Colorectal Cancers" Venkatesh Govindarajan, PhD, Department of Biomedical Sciences.

Creighton University
Internal Grant Application Guidelines
Application Deadline: 4:30 p.m., Monday, April 13, 2015

ELIGIBILITY: Specific eligibility criteria for each internal grant opportunity on the Sponsored Programs Administration website at: http://www.creighton.edu/researchcompliance/grants/internalgrantopportunities.

DEADLINE AND APPLICATION FORMAT: An **electronic pdf copy of each internal application including the Proposal Routing Form** must be submitted to bherr@creighton.edu by **4:30 p.m., Monday, April 13, 2015**. Please submit one pdf document for each application.

SIGNATURES: Internal grant applicants must follow established University approval procedures. The Principal Investigator must complete a Creighton University Proposal Routing Form and obtain the department chairperson's signature before submission to the Sponsored Programs Administration Office. In addition, the Principal Investigator must obtain the signatures of any co-investigators and their department chair. Sponsored Programs Administration will obtain the signature of the Dean and Vice Provost for Research and Scholarship.

PREPARATION OF APPLICATIONS: Total application must include the Creighton University Proposal Routing Form, face page, budget, budget justification, literature cited, a Biographical Sketch for each investigator and no more than 6 pages for the research plan section. Use Arial typeface, a font size of 11 points or larger, and no less than one-half inch margins (top, bottom, left, and right).

FACE PAGE: Use the partially prefilled PHS 398 form located on the Sponsored Programs Administration website at: http://www.creighton.edu/researchcompliance/grants/internalgrantopportunities. Complete a separate face page for each application submitted.

BUDGET PAGES - PHS 398 FORM PAGE 4 and FORM PAGE 5 (Rev 08/2012): Specific budget restrictions for each internal grant opportunity can be located on the Sponsored Programs Administration website at: http://www.creighton.edu/researchcompliance/grants/internalgrantopportunities. See the e-mail instructions for budget restrictions of each internal seed grant program. Complete the PHS398 form page 4 budget page. *(Do not indicate person months for faculty on the budget).*

BUDGET JUSTIFICATION: Describe the specific functions of all personnel. *(Do not indicate person months for faculty on the budget justification).* Provide a complete justification for all non-personnel items requested. No specific form page is required for the budget justification.

PHS 398 BIOGRAPHICAL SKETCH (Rev 08/2012): Provide a biographical sketch for all investigators involved in the proposed project. Use the current PHS398 Biographical Sketch form. A sample Biographical Sketch can be found at: http://grants1.nih.gov/grants/funding/phs398/biosketchsample.pdf.

RESEARCH PLAN : *(No more than 6 pages for the following sections of the Research Plan)*
Please follow the outline below for the narrative of the proposal. This section should include sufficient information needed for evaluation of the project, independent of any other document. Be specific and informative, and avoid redundancies. Discussion of the inclusion of human subjects or animals must be included within the 6 pages of the Research Plan. No abstract is required. There are no specific form pages for the research plan, but use the following format:

1. **Specific Aims:** State concisely the goals of the proposed research and summarize the expected outcomes(s), including the impact that the results of the proposed research will exert on the research field(s) involved. List succinctly the specific objectives of the research proposed, e.g., to test a stated hypothesis, create a novel design, solve a specific problem, challenge an existing paradigm or clinical practice, address a critical barrier to progress in the field, or develop new technology.

2. **Research Strategy:** Organize the Research Strategy in the specified order and using the instructions provided below. Start each section with the appropriate section heading—Significance, Innovation, Approach.

   a. **Significance:**
      - Explain the importance of the problem or critical barrier to progress in the field that the proposed project addresses.
      - Explain how the proposed project will improve scientific knowledge, technical capability, and/or clinical practice in one or more broad fields.
      - Describe how the concepts, methods, technologies, treatments, services, or preventative interventions that drive this field will be changed if the proposed aims are achieved.

   b. **Innovation:**
      - Explain how the application challenges and seeks to shift current research or clinical practice paradigms.
      - Describe any novel theoretical concepts, approaches or methodologies, instrumentation or intervention(s) to be developed or used, and any advantage over existing methodologies, instrumentation or intervention(s).
      - Explain any refinements, improvements, or new applications of theoretical concepts, approaches or methodologies, instrumentation or interventions.

   c. **Approach:**
      - Describe the overall strategy, methodology, and analyses to be used to accomplish the specific aims of the project. Include how the data will be collected, analyzed, and interpreted as well as any resource sharing plans as appropriate.
      - Discuss potential problems, alternative strategies, and benchmarks for success anticipated to achieve the aims.
      - If the project is in the early stages of development, describe any strategy to establish feasibility, and address the management of any high risk aspects of the proposed work.
      - Discuss your plans for potential sources of future support for continuing the research program initiated by this application. Extramural funding agencies to be approached should be specified. In addition, if this research is included in any currently pending external proposal, that proposal should be identified.

LITERATURE CITED: *(Not included in 6 page limitation)*
List all references. Each reference must include the title, names of all authors, book or journal, volume number, page numbers, and year of publication. Be concise and select only those literature references pertinent to the proposed research.

PROJECT START DATE: Grants will be awarded with an approximate start date of July 1, 2015.

CERTIFICATIONS: University procedures for projects involving human subjects, vertebrate animals or biohazardous materials must be observed. Approval must be received prior to the release of funds.

QUESTIONS: If you have any questions, please contact Beth Herr at 402-280-5769 in Sponsored Programs Administration.

Cancer and Smoking Disease Research Development Program
April 2015

#	PRINCIPAL INVESTIGATOR	SCHOOL	DEPARTMENT	CO-INVESTIGATORS	SCHOOL	TITLE	AMOUNT
1	Shana Harrington	Pharmacy	Physical Therapy	None	None	Implementation of a Prospective Surveillance Rehabilitation Model with in a Regional Health System	$ 81,803
2	Deniz Yilmazer -Hanke	Medicine	Biomedical Sciences	None	None	Regulation of ITIH3 by Nicotine and Tobacco Smoke through the CD44 Receptor	$ 120,000
3	Venkatesh Govindarajan	Medicine	Biomedical Sciences	Brian Loggie Peter Thomas Alekha Dash	Medicine Medicine Pharmacy	Acquired Resistance to Targeted Therapy in Mucinous Colorectal Cancers	$ 95,000

Creighton
UNIVERSITY

Sponsored Programs Administration

July 8, 2015

Dr. Venkatesh Govindarajan
Department of Biomedical Sciences
School of Medicine

Dear Dr. Govindarajan:

Thank you for submitting an application to the Cancer and Smoking Disease Research Development Grant Program. We received many impressive and highly competitive proposals. I am pleased to inform you that your application was among the most outstanding applications, and has therefore been selected for funding. Your application committee composite score is 34 and reviewer comments are attached.

I congratulate you on this successful application and look forward to the progression of your research program as you seek to establish extramural funding for this exciting project.

This award will be from July 1, 2015 through June 30, 2016. Your proposal will be funded by the Creighton University LB-595 mechanism in the amount of $60,000. Funding for the second year of your project will be contingent on adequate progress during the initial year of the award.

Please submit a Creighton Budget form to Sponsored Programs Administration as soon as possible so that we can establish a fund number for this award.

I thank you for your time and interest in this program.

Sincerely,

Thomas F. Murray

Thomas F. Murray, Ph.D.
Associate Vice Provost for Research and Scholarship

Cc: Sponsored Programs Administration
Jerrod Lawrence

Location: Criss I, Room 108 Mail: 2500 California Plaza • Omaha, NE 68178
phone: 402.280.2064 • fax: 402.280.4766 • www.creighton.edu/Grants

Creighton University Internal Grant Application

Face Page

1. TITLE OF PROJECT *(Do not exceed 200 characters, including spaces and punctuation.)*

Acquired Resistance to Targeted Therapy in Mucinous Colorectal Cancers

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☐ NO ☒ YES
(If "Yes," Check appropriate box to identify the program to which you are submitting)

Check One

- ☐ **Health Science Strategic Investment Fund Faculty Development Grants**
- ☐ **LB692 - NE Tobacco Settlement Biomedical Research Development New Initiative Grant**
- ☒ **LB595 - Cancer and Smoking Disease Research Program Development Grant**

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR

3a. NAME (Last, first, middle)	3b. DEGREE(S)
Venkatesh Govindarajan	PhD
3c. POSITION TITLE Associate Professor	3d. MAILING ADDRESS *(Street, city, state, zip code)* 253 Criss III, Cancer Center 2500 California Plaza Omaha, NE 68178
3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT Biomedical Sciences	
3f. MAJOR SUBDIVISION	
3g. TELEPHONE AND FAX *(Area code, number and extension)* TEL: 402-280-1819 FAX: 402-280-2960	E-MAIL ADDRESS: g.v@creighton.edu

4. HUMAN SUBJECTS RESEARCH	4a. Research Exempt	If "Yes," Exemption No.
☒ No ☐ Yes	☐ No ☐ Yes	

4b. Federal-Wide Assurance No.	4c. Clinical Trial	4d. NIH-defined Phase III Clinical Trial
00001078	☒ No ☐ Yes	☒ No ☐ Yes

5. VERTEBRATE ANIMALS	5a. Animal Welfare Assurance No.
☐ No ☒ Yes	A3348-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs($)
07/01/2015	6/30/2017	$60,000	$60,000	$95,000	$95,000

9. APPLICANT ORGANIZATION	10. TYPE OF ORGANIZATION
Name: Creighton University Address: 2500 California Plaza Omaha, NE 68178	Public: → ☐ Federal ☐ State ☐ Local Private: → ☒ Private Nonprofit For-profit: → ☐ General ☐ Small Business ☐ Woman-owned ☐ Socially and Economically Disadvantaged
	11. ENTITY IDENTIFICATION NUMBER 1470376583A1
	DUNS NO. 05-330-9332 Cong. District NE-002

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE	13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Beth J. Herr	Name: Beth J. Herr
Title: Director, Sponsored Programs Administration	Title: Director, Sponsored Programs Administration
Address: Creighton University 2500 California Plaza Omaha, NE 68178	Address: Creighton University 2500 California Plaza Omaha, NE 68178
Tel:402-280-5769 FAX:402-280-4766	Tel: 402-280-5769 FAX: 402-280-4766
E-Mail: grantsadmin@creighton.edu	E-Mail: grantsadmin@creighotn.edu

Program Director/Principal Investigator (Last, First, Middle): Govindarajan, Venkatesh

DETAILED BUDGET FOR INITIAL BUDGET PERIOD DIRECT COSTS ONLY	FROM 7/1/2015	THROUGH 6/30/2017

List PERSONNEL *(Applicant organization only)*
Use Cal, Acad, or Summer to Enter Months Devoted to Project
Enter Dollar Amounts Requested *(omit cents)* for Salary Requested and Fringe Benefits

NAME	ROLE ON PROJECT	Cal. Mnths	Acad. Mnths	Summer Mnths	INST.BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Venkatesh Govindarajan PhD	PI	0			0	0	0	0
Brian Loggie MD	Co-I	0			0	0	0	0
Peter Thomas PhD	Co-I	0			0	0	0	0
Alekha Dash PhD	Co-I	0			0	0	0	0
Murali R. Kuracha PhD	Post doc	10.8 3			41,415 42,658	37,274 10,664	10,772 3,175	48,046 13,839
SUBTOTALS →						47,938	13,947	61,885

CONSULTANT COSTS

EQUIPMENT *(Itemize)*

SUPPLIES *(Itemize by category)*
1. Enzymes, Cell fractionation kits, qPCR reagents, Small molecule inhibitors ($3500)
2. Cell culture media, Plastic ware, Chemicals ($2500)
3. Oligonucleotides, Sequencing costs ($1500)
4. Film (Xray, polaroid), Chemiluminescent substrates ($1500)
5. Immunological reagents (antibodies) ($7035)

16,035

TRAVEL

INPATIENT CARE COSTS

OUTPATIENT CARE COSTS

ALTERATIONS AND RENOVATIONS *(Itemize by category)*

OTHER EXPENSES *(Itemize by category)*
Animal purchase and housing

17,080

CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*		$ 95,000
CONSORTIUM/CONTRACTUAL COSTS	FACILITIES AND ADMINISTRATIVE COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		$ 95,000

BUDGET JUSTIFICATION

Venkatesh Govindarajan (GV), Ph.D., (Principal Investigator) will be responsible for oversight and coordination of all aspects of the proposed research. Dr. Govindarajan will guide Murali (post doc) in design of experiments, design of short hairpin RNA, interpretation of data and with submission of manuscripts and abstracts.

Murali R. Kuracha Ph.D., (post doc, 10.8 calendar months (Year 1), 3 calendar months (Year 2)) will be responsible for performing FOXO localization studies (sub-cellular fractionation, immunocytochemistry), short hairpin knockdown of FOXO proteins, qRTPCR assays and western blots to analyze alterations in RTK expression. He will also perform in vivo studies (FOXO knockdown and PI3Ki treatment in MCA mouse models). He will assess escape from adaptive resistance to PI3Ki by analyzing alterations in tumor growth and histopathology. Murali has the necessary experience and expertise in performing cell culture and animal studies. Murali has four publications from the work performed in my lab and two more manuscripts that will be submitted within the next four months.

Other Significant Contributors

Brian Loggie, M.D., a surgical oncologist who specializes in the treatment of peritoneal neoplastic diseases including mucinous colon adenocarcinoma, pseudomyxoma peritonei and peritoneal mesothelioma will lend his clinical expertise and guide design and analysis of the proposed research.

Peter Thomas, Ph.D., a cancer cell biologist, who has over 30 years of experience in studying and working with metastatic colon cancers, will advise us on generation and morphometric analysis of tumors in our xenograft mouse models.

Alekha Dash, R. Ph., Ph.D., a pharmacist, will advise us on the choice of an appropriate dosing regimen for the PI3K inhibitor studies on our mouse models.

Supplies: Laboratory supplies that will be required will include enzymes, cell fractionation kits, qPCR reagents, small molecule inhibitors (PI3Ki) for in vitro studies ($3500); Cell culture media, plastic ware, chemicals ($2500); Oligonucleotides, Sequencing costs ($1500); Film (Xray, Polaroid), Chemiluminescent substrates ($1500); Immunological reagents (antibodies) ($7035).

Animal costs ($17,080).

Mouse (*Mus musculus*) costs include the cost of purchase of athymic nude mice and the cost of maintenance of these mice for the duration of the studies. Based on our power calculations (described in grant application), we will need an n=8 for each experimental and control groups. A total of 192 mice will be used (6 treatment groups (listed in Table in the grant application) x 2 doses x 8 mice/treatment group/cell line x 2 cell lines (LS174T, RW7213) = 192 mice). Total cost of purchase of nude mice (athymic nude-Foxn1^{nu} from Harlan Labs strain code 490) will be $80/mouse x 192 mice = $15,360.

The cost of maintenance of mice for our animal studies is $1720.32. This number was calculated as follows; we will maintain 192 mice for 4 weeks (3 weeks for the studies + one week for contingency). Assuming a per diem rate of $0.32/mouse/day, the projected animal costs for these studies would be $1720.32 (192 mice x 4 weeks x 7 days/week x $0.32/mouse/day);

Therefore, total animal costs will be $17,080 ($15,360 (purchase costs) + $1720.32 (maintenance))

1. SPECIFIC AIMS. Mucinous colorectal adenocarcinoma (**MCA**) is a colorectal cancer (**CRC**) subtype characterized by substantial amount of extracellular mucin (>50% of the tumor) with a high incidence of peritoneal metastases and poor prognosis (1). MCAs are associated with poorer response to adjuvant chemotherapy and chemoradiotherapy (1-3). Treatment options for MCA are limited to cytoreductive surgery and intraperitoneal/systemic chemotherapy. Not all patients are candidates for, or benefit from, these procedures and the grim reality is that majority of the patients with peritoneal dissemination will have recurrent disease and die. Therefore, targeted therapies that are more effective and less toxic than current approaches are badly needed. However, a significant problem with targeted therapeutics in general is the adaptive resistance that develops in response to drug treatment. Knowledge of cellular by-pass mechanisms that mediate adaptive resistance is critical to achieve effective and durable therapeutic response. The ***objective*** of this application is to define the molecular basis of adaptive resistance that develops in response to phosphoinositide 3 kinase (PI3K) inhibition in MCA cancer cells.

Our *preliminary studies* show; a) higher mutation rates in RAS/RAF/ERK and PI3K/AKT pathway effectors in mucinous than in nonmucinous CRCs, b) this higher pathway upregulation is associated with cellular dependence ('addiction'), c) PI3K single agent inhibition triggers pathway downregulation but also leads to nuclear enrichment of the transcription factor, FOXO3A, with a concomitant increase in receptor tyrosine kinase (RTK) expression, activation and development of adaptive resistance and d) this adaptive resistance could be overcome by combined inhibition of PI3K and of a downstream effector of RTKs, MEK. The proposed studies will test the ***central hypothesis*** that FOXO-induced expression of RTKs (ErbB2, ErbB3, insulin receptor (IR), insulin growth factor receptor 1 (IGFR1), Ephrin receptor A7 (EphA7) and Ephrin receptor A10 (EphA10)) is a key mediator of adaptive resistance that develops in response to PI3K inhibition (PI3Ki) in MCA cancers. We will test our hypothesis by pursuing the following specific aim;

Aim. Define the roles of FOXO proteins in mediating adaptive resistance to PI3Ki in vitro and in vivo.

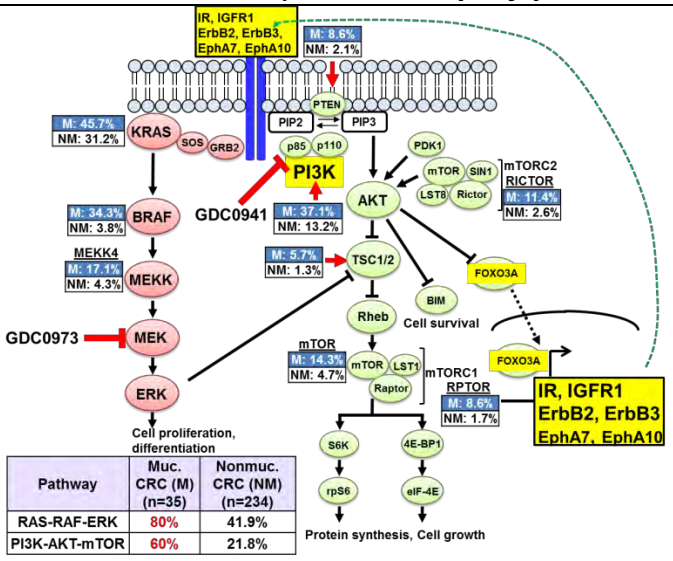
Our *working hypothesis* is that reduction in phospho-AKT levels in response to PI3K inhibition leads to a) nuclear localization of FOXO proteins which in turn leads to b) transcriptional upregulation of RTKs (ErbB2, ErbB3, IR, IGFR1, EphA7 and EphA10) whose expression and activation leads to adaptive resistance to PI3K inhibition. These hypotheses will be tested in vitro (sub-cellular fractionation studies, shRNA knockdown approaches) and in vivo (xenograft mouse models).

Currently, KRAS mutations limit the use of EGFR inhibitor therapy for metastatic CRCs (4, 5). The substantial percentage of activating mutations in the KRAS and PI3K pathway effectors in mucinous CRCs and associated addiction to pathways mediated by these effectors logically define targets for treatment. Successful completion of the proposed studies will elucidate the mechanistic details of cellular by-pass mechanisms that mediate adaptive resistance to PI3K inhibitors and provide a rational basis for PI3Ki and MEKi combination therapy for treatment of these mucinous malignancies. As these signaling pathways are frequently hyperactivated and are drivers of neoplastic growth in other cancers, these studies will have broad clinical translational impact.

2. RESEARCH STRATEGY

A. SIGNIFICANCE. A significant proportion of sporadic CRCs (10-15%) include the mucinous subtype (6). Even among hereditary nonpolyposis colorectal cancers (HNPCC or Lynch Syndrome), a substantial fraction (20-25%) is mucinous (7, 8). Though MCAs share certain features with nonmucinous CRCs, they are considered to be clinically, morphologically and molecularly distinct as they show a different spectrum of genetic alterations (MSI-H, lower p53, high MUC2) and exhibit more aggressive behavior (more prone to peritoneal dissemination and lymph node involvement) (9-11). MCAs are associated with poorer response to adjuvant chemotherapy and chemoradiotherapy (12-14). Systemic chemotherapy is not standardized for peritoneal carcinomatosis and aggressive cytoreductive surgery (CRS) (reduction of all macroscopic lesions) in conjunction with hyperthermic intraperitoneal chemotherapy (HIPEC) (circulation of heated chemotherapeutic agents within the peritoneal cavity) has been the only treatment intervention shown to improve patient outcomes (18). Nevertheless, not all patients are candidates for this treatment and the majority will have persistent or recurrent disease. Our preliminary data indicate that MCA cells, though initially responsive to PI3Ki treatment, later acquire resistance. ***The contributions of the proposed studies would be*** to define the role of FOXO proteins in mediating adaptive resistance to PI3K inhibitor therapy. Currently, identification of KRAS (and BRAF) mutations in patients with metastatic CRC removes selective EGFR inhibition (Erbitux) as a treatment option (4, 5). As there are no diagnostic and/or treatment interventions specific to MCA, these studies have the potential to alter clinical practice.

B. INNOVATION. 1) *Approach.* Existent literature on mucinous colorectal adenocarcinomas fall into two broad categories; A) clinico-correlative studies associating either mutations in oncogenes/tumor suppressors (5) or the effectiveness of chemotherapeutic agents/methods to patient outcomes (2, 3, 6) and B) microarray (7) or nextgen sequencing efforts (8, 9) to catalog genetic alterations in cancer genes. Though these studies have yielded important new information, a significant limitation is that they are largely correlative: they have not established causal connections between the observed genetic alterations and neoplastic growth nor have they identified novel targets for therapy. The studies we have proposed here will, for the first time, test the possibility of, and provide a rational basis for, targeted treatment intervention. **2) *We are uniquely positioned to undertake these studies because*** a) we have access to the MCA patient population through our peritoneal neoplastic disease program (directed by co-I, Dr. Brian Loggie, surgical oncologist), b) we have a strong translational working group that includes a surgical oncologist (BL), a pathologist (PS), a pharmacist (AD) and a well experienced cancer biologist (PT), c) the small molecule inhibitors we are using are already in clinical trials for treatment of other cancers and d) pre-clinical MCA patient-derived xenograft mouse models have been generated and the effectiveness of our inhibitors in reducing tumor growth in these models will be tested in the future after characterization of these models (these studies are not a part of this grant proposal).



Pathway	Muc. CRC (M) (n=35)	Nonmuc. CRC (NM) (n=234)
RAS-RAF-ERK	80%	41.9%
PI3K-AKT-mTOR	60%	21.8%

Fig 1. Bioinformatic analysis of TCGA CRC datasets reveal higher mutation rates for effectors of the RAS (colored red) and PI3K pathways (colored green) in mucinous (M) than in nonmucinous (NM) CRCs. Mutation percentages are shown in boxes adjacent to each effector. Effectors relevant to the proposed studies are highlighted in yellow.

C. APPROACH

Higher mutation rates in mucinous CRCs. Our bioinformatic analyses (using cBioPortal (10)) of the cancer genome atlas (TCGA) CRC datasets (11) showed higher mutation rates in mucinous than in nonmucinous CRCs for effectors of RAS-RAF-MEK-ERK (80% vs. 41.9%) and PI3K-AKT-mTOR (60% vs. 21.8%) pathways (box in Fig. 1). These increased mutation rates suggested the importance of these pathways to oncogenesis of these mucinous neoplasms.

PI3K and MEK inhibition in vitro. To assess whether sustained signaling through these pathways are necessary for survival of these tumors, we attenuated signaling mediated through two key effectors, PI3K and MEK, using two small molecule inhibitors GDC0941 and GDC0973 (Genentech) respectively. GDC0941 (**Pictilisib**) is a potent inhibitor of class I PI3K isoforms (α (IC50: 5 nM), β (IC50: 27 nM), γ (IC50:14 nM), δ (IC50: 7 nM)) and shows strong selectivity (tested against mTOR and 228 other kinases (12)). GDC0973 (**Cobimetinib**) is a potent and highly selective MEK inhibitor (IC50: 4.2 nM against MEK1 and >100 fold selectivity to MEK than 100 other serine-threonine and serine kinases (13)). These inhibitors have been shown to be effective on a broad panel of cancer cell lines and cell line-derived mouse models (13-15) and are in early stage clinical trials for a variety of cancers (Clinicaltrials.gov). To test their effectiveness in MCA, we chose 3 MCA cell lines, LS174T (KRAS G12D, PI3K H1047R), RW7213 (KRAS G12C, PI3K WT) and RW2982 (KRAS Q61R, PI3K WT). Cell viability assays for the two inhibitors showed that all the three MCA lines were sensitive to the MEK inhibitor (IC50s for LS174T, RW7213, RW2982 were 0.28 μM, 0.23 μM and 0.44 μM respectively) (for brevity, dose response curves not shown). Interestingly, LS174T and RW7213 cell lines were sensitive to the PI3K inhibitor at 48 hours but by 72 hours had become resistant (we deem a cell line to be resistant if we cannot fit the data (10 data points for each assay, n=6 for each point) to a 4 parameter logistic/sigmoidal dose response curve with an r^2>0.9 and a negative Hill slope) (the cell line RW2982 was resistant by 48 hours). The loss of sensitivity in

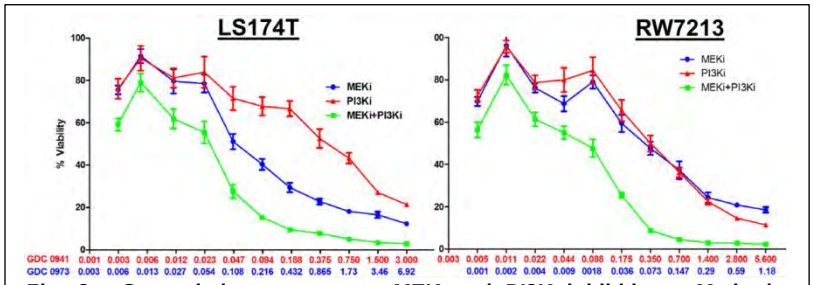


Fig. 2. Synergistic response to MEK and PI3K inhibition. Methods: LS174T and RW7213 lines were treated with MEKi alone (blue), PI3Ki alone (red) or a combination of the two inhibitors in a fixed ratio (green) for 72 hours (n=6 for each data point). Error bars are standard deviations. X axis: concentration of inhibitors in μM. Y axis: % viability normalized to vehicle treated controls.

LS174T and RW7213 suggested the development of adaptive resistance to the PI3K inhibitor in these lines. Though resistance developed to PI3K inhibition by 72 hours, combined treatment with both PI3K and MEK inhibitors showed a synergistic loss of viability in these cell lines (Fig. 2). ***These results indicated that the adaptive resistance that develops in response to PI3K inhibition can be overcome by combined treatment of PI3K and MEK.*** This outcome suggested the possibility that increased MEK-ERK signaling likely drives adaptive resistance. Consistent with this hypothesis, an increase in phosphorylated ERK (pERK) was seen at 72 but not at 48 hours (compare pERK bands marked by red and green arrows respectively in Fig. 3). Concomitant increase in pAKT, a downstream effector of PI3K, was also seen at 72 hours (compare pAKT bands marked by red and green arrows in Fig. 3) suggesting a rebound activation of the PI3K-AKT pathway in spite of the presence of the PI3K inhibitor. Similar alterations were also seen in the other cell line RW7213 (for brevity, data not shown). ***Taken together, these results correlated the development of adaptive resistance to elevation in pERK and pAKT signaling.***

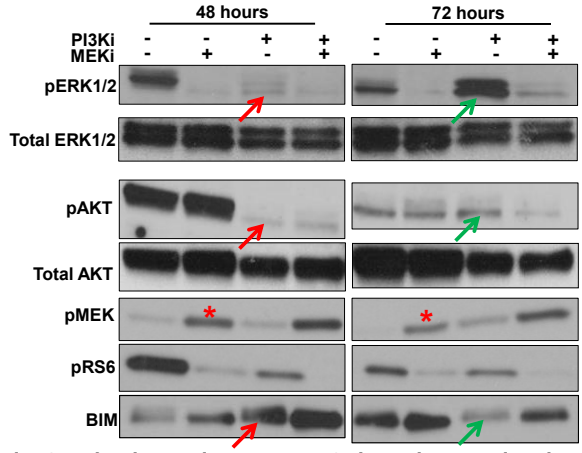


Fig. 3. Adaptive resistance to PI3Ki can be correlated to increased pERK, pAKT and reduced BIM. Methods: LS174T cells were treated with MEKi and PI3Ki alone or in combination for 48 and 72 hours, proteins isolated, resolved, blotted and probed with the antibodies listed to the left of each blot. Asterisks mark increased phosphorylation of MEK in response to MEK inhibition. This paradoxical rebound phosphorylation in MEK is due to relief of negative feedback inhibition of MEK by ERK, a phenomenon well documented in the literature (4).

Cytotoxic response is due to induction of apoptosis. Associated with loss of cell viability in response to MEK and PI3K inhibition was a proportionate increase in cleaved PARP, a marker of apoptosis (for brevity, data not shown). A critical mediator of apoptosis in response to tyrosine kinase inhibitors (TKI), MEK or PI3K inhibition in oncogene-addicted cancer cells is the BH3-only protein, **BIM** (B-cell lymphoma 2 interacting mediator of cell death). BIM binds and inactivates anti-apoptotic BCL-2 family members, Mcl-1 and Bcl-2, thereby countering their pro-survival effects and also activates the pro-apoptotic proteins Bak and Bax to promote cell death (16). We chose to examine BIM protein levels because a) BIM is the only protein (with the exception of PUMA) that has been shown to bind with high affinity to all Bcl2 family members (17, 18), b) BIM is rate limiting for apoptosis (19), c) BIM is a key mediator of apoptosis in response to targeted therapies (20-24), and d) BIM protein stability is negatively regulated by ERK phosphorylation (25). In our MCA cell lines, BIM expression was elevated in response to MEKi and PI3Ki single agent treatment and showed a more pronounced increase in response to combined treatment (Fig. 3). *In addition, increased cell viability and development of adaptive resistance in response to PI3K inhibitor treatment by 72 hours could be correlated to a reduction in BIM expression (Fig. 3, bottom panel compare bands marked by green and red arrows).* This result suggests a potential utility for BIM as a predictive biomarker for PI3K inhibitor therapy.

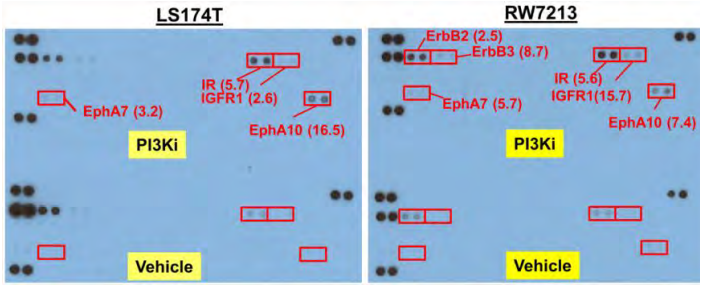


Fig 4. Increased RTK phosphorylation upon prolonged exposure to PI3K inhibitors. Methods: 72 hours after PI3Ki treatment, LS174T and RW7213 cell lysates were incubated with anti-phospho-tyrosine antibody and RTK profiler antibody array (R&D biosystems) containing 49 RTKs. Pixel densities were quantified by image analysis software. The upper half shows alterations in the PI3Ki samples and the lower half shows respective vehicle-treated controls. The red boxes mark the RTKs that were elevated and the numbers indicate fold elevation relative to controls.

Relief of negative feedback of RTKs drives adaptive resistance. The compensatory activation of MEK-ERK signaling in response to PI3K inhibition led us to explore activation of RTKs that are upstream initiators of these signaling pathways. For this, we used a phospho-RTK array from R&D (Array Profiler) that allows us to interrogate the activation i.e. phosphorylation status, of 49 RTKs simultaneously. In LS174T and RW7213 cell lines, 72 hours after PI3K inhibitor treatment, increases in phosphorylated insulin receptor (5.7 and 5.6 fold), IGFR1 (2.6 and 15.7 fold), EphA10 (16.5 and 7.4 fold) and EphA7 (3.2 and 5.7 fold) were seen (Fig. 4). In RW7213, additional increases in ErbB2 (2.5 fold) and ErbB3 (8.7 fold) were also seen (Fig. 4). ***These results correlated the development of adaptive resistance to increased activation of RTKs.***

FOXOs. FOXO proteins are winged helix/forkhead box transcription factors that have been shown to regulate a variety of cellular processes including cell survival, death, metabolism and stress response (26). There are four principal members of the FOXO subfamily- FOXO1, FOXO3, FOXO4 and FOXO6 (27). Of these, FOXO6 is neuron-specific and the other FOXOs are expressed in most tissues (27). Of relevance to our studies is the fact that FOXO proteins are negatively regulated by PI3K-AKT signaling; activated AKT phosphorylates FOXOs on conserved serine and threonine residues and this leads to increased binding of FOXO to the 14-3-3 protein and nuclear exclusion of FOXO thereby preventing FOXO from transcriptionally activating its downstream targets (28). FOXOs have been linked to adaptive resistance to AKT inhibition in breast cancer cell lines (29). To test whether FOXO proteins are relevant to acquired resistance to PI3Ki treatment in MCA cells, we examined alterations in FOXO3A localization by immunocytochemistry. At 48 hours, when MCA cells are sensitive to PI3Ki, FOXO3A was mostly found in the cytoplasm (Fig. 5C, green) similar to vehicle (Fig. 5A, green)- and MEKi (Fig. 5B, green)-treated cells. In contrast, at 72 hours, when MCA cells develop resistance to PI3Ki, FOXO3A protein was enriched in the nucleus in PI3Ki-treated cells (Fig. 5F, arrows, note that the red color that clearly marks the nuclei in other panels is obscured by the green FOXO3A staining). The cytoplasmic staining intensity of FOXO3A appeared similar between the different treatment groups (Fig. 5D-F). Similar results were seen in RW7213 cells (for brevity, data not shown). ***These results correlate FOXO3A nuclear enrichment to the acquisition of adaptive resistance to PI3K single agent inhibition.*** The proposed studies a) will examine the alterations in sub-cellular localization of other FOXOs (FOXO1, FOXO4) in response to PI3Ki treatment and b) test whether FOXOs are necessary for adaptive resistance to PI3Ki treatment.

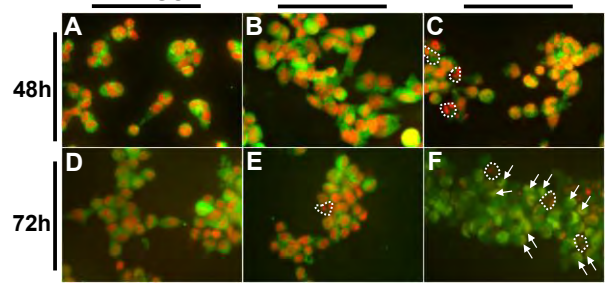


Fig. 5. FOXO3A nuclear localization 72 hours after PI3Ki treatment. Methods. 48 or 72 hours after PI3Ki treatment, LS174T cells were fixed, incubated with an anti-FOXO3A antibody and the antigen-antibody complex was detected by fluorescence. Nuclei are pseudocolored red and FOXO3A, green. Nuclear enrichment of FOXO3A is seen at 72 hours (F, nuclei appear yellow due to the red-green overlap (arrows) unlike in other panels where the red nuclei are clearly visible) but not 48 hours (C) after PI3Ki treatment. Circled cells in C, E and F are likely dead or dying cells with fragmented nuclei.

Summary. Taken together, these results collectively support ***a model (Fig. 1)*** for adaptive resistance to PI3K inhibition in MCA cells that involves the following sequence; 1) Exposure to PI3K inhibitor initially reduces AKT signaling; 2) this reduction leads relief of inhibition and nuclear enrichment of FOXO3A; 3) this in turn, allows FOXO3A-mediated transcriptional activation of RTKs including IR, IGFR1, EphA10, EphA7, ErbB2 and ErbB3; 3) increased expression of these RTKs leads to their increased activation (likely through receptor homo- or heterodimerzation); 4) RTK activation stimulates rebound activation of RAS(wildtype)-RAF-MEK-ERK and PI3K-AKT-mTOR pathways; 5) this increase leads to decreased BIM levels and 6) decrease in BIM tips the cellular balance in favor of pro-survival signals leading to increased cell viability and ultimately, acquisition of resistance. Our on-going studies are designed to test the predictions of this model. [Note: Our KRAS knockdown studies show that mutant KRAS-mediated signaling through MEK and PI3K are necessary to sustain MUC2 (predominant component of mucin) production in MCA (manuscript in preparation). This ties the activation of these pathways to the mucin overexpression phenotype in MCA. Down regulation of MUC2 production could be a beneficial side effect of MEK and PI3K inhibitor therapy although decreased proliferation and increased apoptosis are the intended consequences]. ***The scope of this grant application however, is restricted to the analysis of the roles of FOXO proteins in mediating adaptive resistance to PI3K inhibition.***

Aim. Define the roles of FOXO proteins in mediating adaptive resistance to PI3Ki in vitro and in vivo.

1.1. *FOXO localization studies.*

A. ICC. In addition to FOXO3A, we will also examine sub-cellular distribution patterns of FOXO1 and FOXO4 by immunocytochemistry using anti-FOXO1 and anti-FOXO4 antibodies (Cell Signaling, Cat#s 2880, 9472) at 48 and 72 hours of PI3Ki treatment of LS174T and RW7213 cells (our qRTPCR studies indicate that FOXO1 and FOXO4 are expressed at low levels in these two cell lines). Digital images will be captured by confocal microscopy (Creighton Microscopy Core Facility). The other FOXO, FOXO6, is neuron-specific and is therefore, not considered here.

B. Fractionation studies. We will confirm our ICC results biochemically by sub-cellular fractionation studies. We will separate nuclear from cytoplasmic fractions by using NE-PER nuclear and cytoplasmic extraction kit

(Pierce, Rockford, IL, Cat#78835) that employs a detergent based separation method (instead of the classical homogenization and differential ultracentrifugation procedures which are lengthier) to isolate proteins from nuclear and cytoplasmic fractions. The proteins will be resolved by SDS-PAGE, blotted and probed with anti-FOXO1, 3A or 4 antibodies. The purity of the nuclear and cytoplasmic fractions will be confirmed by western blots of Oct-1 (nuclear) and heat shock protein 90 (HSP90) (cytoplasmic) (based on manufacturer's recommendations).

1.2. ***FOXO inactivation studies.*** The hypothesis that FOXO3A nuclear localization is necessary for transcriptional upregulation of RTKs, will be tested by inactivation of FOXO3A function by short hairpin RNA (shRNA) targeted against FOXO mRNA (against sequences common to all three FOXOs). MCA lines, LS174T and RW7213, will be infected with the pINDUCER lentiviral vector (30) containing the FOXO shRNA. An advantage in using this vector is that it allows inducible expression of shRNA by addition or withdrawal of doxycycline (DOX) (Fig. 6). As FOXO proteins may regulate other essential functions within the cell, a non-inducible (or a constitutive) FOXO knockdown could be lethal. An inducible knockdown approach will therefore, be useful in this regard. Another advantage of the pINDUCER system is the coordinate expression of a fluorescent reporter (tRFP) along with the shRNA (Fig. 6). This allows, by FACS sorting, the separation of those cells that show the highest shRNA expression from a polyclonal population of lentivirus-infected cells. The sorted cells can then be expanded and stable lines established. Thus, this approach helps achieve an efficient, reliable and cleaner knockdown of target mRNAs than other knockdown methods. We have successfully used the pINDUCER vectors in our lab to knockdown a number of genes including KRAS, MUC2, SPDEF and Agr2 in LS174T and RW7213 cells. We routinely get 70-80% knockdown using this approach. Stable lines will be established from FACS sorted tRFP^{hi}, FOXO shRNA-containing cells and FOXO knockdown will be confirmed by qRTPCR assays and western blots. These lines will then be used for downstream applications (1.3) and for our in vivo studies (1.4). Non-silencing shRNA controls will be included. PI3Ki (GDC0941, Genentech) dose response curves will be generated to determine whether MCA cell lines escape adaptive resistance upon FOXO knockdown at 72 hours. In addition, as shown in Fig. 3, we will perform western blots to correlate escape from adaptive resistance to alterations in levels of phospho-ERK, phospho-AKT and BIM.

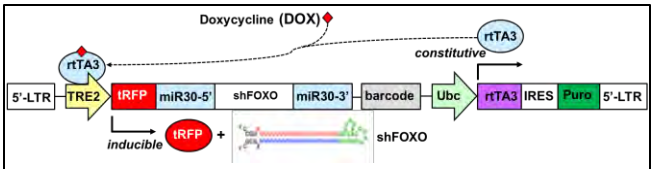


Fig. 6. pINDUCER lentiviral vector (adapted from Meerbrey et al. 2011)

1.3. ***FOXO-mediated RTK expression.*** The hypothesis that FOXO proteins are necessary for transcriptional induction of RTK expression in response to PI3Ki treatment will be tested by qRTPCR assays. LS174T and RW7213 cells will be treated with DOX to induce FOXO knockdown and treated with PI3Ki for 72 hours, RNA isolated, reverse transcribed and the cDNA used for qRTPCR of RTKs, ErbB2, ErbB3, IR, IGFR1, EphA7 and EphA10. Reactions will be performed in triplicate. RTK expression levels will be quantified and compared to untreated and vehicle-treated controls. Western blots will be performed to assess alterations in the RTK protein expression upon FOXO knockdown using antibodies to these RTKs.

Expected outcomes. **1.1. A & B.** Based on our FOXO3A ICC results (Fig. 5), we expect FOXO3A to be enriched in the nuclear fraction at 72, but not 48, hours after PI3Ki treatment. As FOXO1 and FOXO4 are also known targets of PI3K signaling, we would expect similar nuclear enrichment of these proteins as FOXO3A. **1.2.** We expect FOXO knockdown-MCA cells to escape adaptive resistance that would otherwise develop at 72 hours after PI3Ki treatment. We also expect a corresponding reduction in phosphorylation of pERK and pAKT and therefore, a reduced (or absent) rebound activation of these effectors. If these studies are successful then, we will knockdown FOXO1, FOXO3A and FOXO4 individually using the same pINDUCER lentiviral vectors. **1.3.** FOXO knockdown is expected to result in decreased RTK (ErbB2, ErbB3, IR, IGFR1, EphA7 and EphA10) mRNA and protein expression by 72 hours of treatment with PI3Ki.

Potential Problems & Alternative Strategies. We do not anticipate any problems in performing FOXO protein localization studies (1.1A, B). However, if the reagent based method for separation of nuclear from cytoplasmic fractions does not work as planned, then we will use the old-fashioned homogenization, differential ultracentrifugation methods. We do not anticipate any problems with the FOXO knockdown studies (1.2) using the pINDUCER vectors because, as stated earlier, this is an established procedure in the lab. However, in the unlikely event that these do not work as planned, we will inactivate FOXO function by expressing a dominant negative FOXO3A that contains the N-terminal DNA-binding domain but lacks the C-terminal transactivation domain (Addgene, Cat#1796) (31). As this mutant can bind to DNA, it would compete with endogenous wildtype FOXO3A in binding to its downstream target sequences but as it lacks a transactivation domain,

cannot activate transcription. As controls, we will also express a constitutively active version of FOXO3A that has the Ser253 mutated to alanine (Addgene, Cat#8349) (32). Mutation of the Ser253 residue would allow it to escape phosphorylation by AKT and therefore, would be constitutively active. These constructs will be transfected into LS174T and RW7213 cells and treated with PI3Ki for 72 hours (with the inclusion of vehicle treated controls). These plasmids are tagged with HA thereby enabling confirmation of expression of these mutant proteins by western blotting and probing with anti-HA antibodies. A disadvantage with this strategy is that we have to target the FOXO proteins individually and cannot knockdown all the three FOXOs at the same time. Nonetheless, at the end of the funding period, we will have an unambiguous answer to whether or not FOXOs mediate adaptive resistance to PI3Ki treatment in MCA cells.

1.4. ***In vivo studies.*** Based on our preliminary in vitro data *our working hypothesis* is that inhibition of FOXO function in vivo will be sufficient to alleviate adaptive resistance to PI3K inhibition and allow for effective therapeutic response. For these studies, we will subcutaneously inject, into 4-6 week old nude mice, LS174T and RW7213 cells carrying the pINDUCER vector with the FOXO shRNA. Once the tumors have reached 100 mm^3, mice will be split into 6 groups (see **Table**). The drug GDC0941 (PI3Ki) will be given orally, once daily (QD) for 21 days. This dosing regimen was chosen based on a recently published study where this inhibitor was used and shown to be effective on a variety of cancer cell line-derived xenografts (13). This inhibitor is well tolerated by mice. Expression of shRNA in the '+DOX' treatment groups will be induced by replacement of regular feed with DOX-containing special feed (Harlan labs, Cat#TD.01306). An n=8 will be used for each group. This number was chosen by a power analysis that was performed using standard deviations reported by Choudry et al. (2012) where similar xenografts have been described (Choudry et al., 2012). In this study, the mean tumor volume and standard deviation in xenograft nude mice were reported to be 2998 mm^3 and 1109 mm^3 respectively. An n=8 will give us 80% power to detect a 55% difference in mean tumor volume between experimental and control groups with an α=0.05 (two tailed). A total of 192 mice will be used (6 treatment groups (listed in Table below) x 2 doses x 8 mice/treatment group/cell line x 2 cell lines (LS174T, RW7213) = 192 mice). Tumor dimensions will be measured to calculate tumor volume and tumor growth rates will be compared using ANOVA. Tumor growth inhibition will be calculated as a percentage of area under the fitted curve (AUC) for the respective dose group per day in relation to vehicle-treated controls (13). At the end of 21 days, mice will be sacrificed, necropsied and gross morphological, histopathological and immunohistochemical (cytokeratin 20, cytokeratin 7, carcinoembryonic antigen (CEA) (expressed in human MCAs), Cdx2 (a transcription factor necessary for MUC2 expression), MUC2, MUC5AC, MUC6 (mucins expressed in human MCAs) and Ki-67 (proliferation marker) alterations in MCA tumors will be assessed.

Cell line	Arm
LS174T	Vehicle (DMSO) QD
RW7213	PI3Ki (GDC0941) 60 or 120 mg/kg QD
	PI3Ki (GDC0941) 60 or 120 mg/kg QD + Non sil. shRNA (-DOX)
	PI3Ki (GDC0941) 60 or 120 mg/kg QD + Non sil. shRNA (+DOX)
	PI3Ki (GDC0941) 60 or 120 mg/kg QD + FOXO3A shRNA (-DOX)
	PI3Ki (GDC0941) 60 or 120 mg/kg QD + FOXO3A shRNA (+DOX)

Expected outcomes. Based on our in vitro data, we expect the MCA cell line-derived mouse models to also develop adaptive resistance to PI3Ki treatment. Therefore, we do not expect a significant difference in tumor growth rates between vehicle- and PI3Ki-treated mice. However, we expect this resistance to be substantially reduced (or abolished) upon FOXO knockdown. We expect a corresponding reduction in cytokeratin 20, cytokeratin 7, carcinoembryonic antigen (CEA), Cdx2, MUC2, MUC5AC, MUC6 levels and Ki-67 positive cells.

Potential Problems & Alternative Strategies. We do not anticipate any difficulties in performing these studies. The post doc in the lab (Murali Kuracha) is trained in performing s.c. injections and oral gavage. We have obtained, through a material transfer agreement, GDC0941 from Genentech in sufficient quantities to perform the experiments. In the unlikely event that the proposed studies do not work as anticipated, we will alter the dosing regimen (alter strength or duration of inhibitor treatment) (Dr. Alekha Dash, Pharmacist, will lend his expertise to this part of the project) and perform our studies.

Potential sources of future support. Our recent R03 grant application to the NCI received a score of 33 (R03s are not percentiled) and is under funding consideration (no overlap with current proposal). This grant will likely need to be resubmitted (October 2015). The proposed studies in addition to data from our ongoing studies should allow us to submit an R01 grant application in February 2016. In addition, we will submit grant proposals to other agencies such as National Organization of Rare Disorders (NORD) that fund innovative cancer research.

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9. Sio TT, Mansfield AS, Grotz TE, Graham RP, Molina JR, Que FG, et al. Concurrent MCL1 and JUN amplification in pseudomyxoma peritonei: a comprehensive genetic profiling and survival analysis. J Hum Genet. 2014;59(3):124-8. PMCID: 3973125.
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11. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487(7407):330-7. PMCID: 3401966.
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15. Wallin JJ, Guan J, Prior WW, Lee LB, Berry L, Belmont LD, et al. GDC-0941, a novel class I selective PI3K inhibitor, enhances the efficacy of docetaxel in human breast cancer models by increasing cell death in vitro and in vivo. Clin Cancer Res. 2012;18(14):3901-11.
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28. Eijkelenboom A, Burgering BM. FOXOs: signalling integrators for homeostasis maintenance. Nat Rev Mol Cell Biol. 2013;14(2):83-97.
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BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Venkatesh Govindarajan	Associate Professor
eRA COMMONS USER NAME (credential, e.g., agency login) govindar	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Birla Institute of Technology & Science, Pilani, India	M.Sc. (Hons.) & M.M.S (Master of Management Studies)	08/1987-05/1992	Biological Sciences & Management Studies
University of Houston, Houston, Texas	Ph.D.	08/1992-08/1997	Developmental/Molecular Biology
Baylor College of Medicine, Houston, Texas	Postdoctoral Associate	09/1997-10/2002	Developmental/Molecular Biology

A. Personal Statement

I have been working in the area of growth factor signaling and developmental biology since my doctoral, postdoctoral and early years as an independent investigator. At Creighton, my laboratory has analyzed how the fibroblast growth factor (FGF) signaling pathway regulates differentiation of ocular tissues. By generation of transgenic and knockout mice with impaired FGF signaling in ocular tissues, we have shown that FGF signaling is critical for lens, corneal and lacrimal gland differentiation. We have also shown that downstream effectors and modulators of FGF signaling including Ras and Sproutys regulate early specification of ocular tissues (these were NIH-funded studies with me as the PI). In 2012, I switched fields from developmental to cancer biology. I joined a translational working group that works on peritoneal neoplastic diseases including MCA, PMP and peritoneal mesotheliomas. In addition to myself, this group includes a surgical oncologist (Dr. Brian Loggie), a cell biologist with considerable experience in colon cancers (Dr. Peter Thomas), a pathologist (Dr. Poonam Sharma), a radiologist (Dr. Gopi Sirineni) and a pharmacist (Dr. Alekha Dash). This is an active working group with weekly meetings, shared co-authorship in papers (three in 2013, two in 2014), a book chapter (2014), abstracts and posters at national and international meetings (ten in 2012-2014). Recently, our group has identified mutations in the KRAS gene in a significant proportion of the PMP patients, revised the classification of PMPs based on patient outcomes and profiled chemokine/cytokine expression in the peritoneal microenvironment (all published). Our most recent work shows that mutant KRAS regulates MUC2 expression through engagement of both RAS and PI3K-driven pathways (funded by the NORD foundation). The techniques proposed in this grant application have been established and are routinely used in our lab. We have also assembled all the tools and reagents (cell lines and small molecule inhibitors) we will need for our work. *Thus, my experience in the field of signaling, clinical and translational expertise of my colleagues and the unique clinical environment at Creighton places me in a strong position to successfully lead the proposed project.*

B. Positions and Honors.

Professional Experience

1992-1997 Teaching Assistant, Department of Biology, University of Houston, Houston, TX.
1997-2002 Postdoctoral Associate, Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX.
2002-2004 Instructor, Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX.
2004-2009 Assistant Professor, Department of Surgery, Creighton University, Omaha, NE.

2009-2013 Associate Professor, Department of Surgery, Creighton University, Omaha, NE.
2013- Associate Professor, Department of Biomedical Sciences, Creighton University, Omaha, NE.

Research Experience

1992-1997 Graduate Student, Department of Biology, University of Houston, TX
Thesis Advisor: Dr. Craig R. Tomlinson
Thesis Research: Extracellular matrix and growth factor interactions during sea urchin embryogenesis.

1997-2002 Postdoctoral Associate, Department of Molecular & Cellular Biology, Baylor College of Medicine, Houston, TX
Advisor: Dr. Paul A. Overbeek
Research: Analyses of FGF signaling in lens, lacrimal gland and skeletal development.

Honors and Awards

1994, 1996 Department of Biology Travel Award, Sea Urchin Meeting IX and X, 1994 and 1996.
1993, 1996 Department of Biology Travel Award, American Society for Cell Biology Meeting
1996 Predoctoral Travel Award, American Society for Cell Biology Meeting, 1996.
1995, 1996 Sigma Xi Grant-in-Aid of Research (two awards) 1995 and 1996
1996 Best Graduate Student Presentation, Southwest Regional Developmental Biology Meeting, TX
1997 Sigma Xi Graduate Student Achievement Award for Research Excellence, 1997.
2000 Gordon Conference on Visual System Development Travel Fellowship, 2000
2003 National Eye Institute Travel Fellowship, Association for Research in Vision and Ophthalmology
2004 Protein Purification and Characterization Course Tuition & Travel Scholarship, Cold Spring Harbor, NY
2009 Young Investigator Award, Creighton University School of Medicine

National Peer Review Panels

National Institute of Health (NIH) Oral, Dental and Craniofacial Sciences (ODCS) Study Section (ad hoc reviewer)

Journal Referee

Reviewed manuscripts for *Developmental Biology*, *Mechanisms of Development*, *Journal of Anatomy*, *Peptides*, *FEBS Letters*, *BMC Developmental Biology*, *Molecular Vision, Experimental Eye Research, Breast Cancer Research and Treatment*

C. Selected peer-reviewed publications

Most relevant to the current application (from a total of 25 peer reviewed articles)

1. Shetty S, Thomas P, Ramanan B, Sharma P, **Govindarajan V**, Loggie B. Kras mutations and p53 overexpression in pseudomyxoma peritonei: association with phenotype and prognosis. J Surg Res. 2013;180(1):97-103.
2. Shetty S, Natarajan B, Thomas P, **Govindarajan V**, Sharma P, Loggie B. Proposed classification of pseudomyxoma peritonei: influence of signet ring cells on survival. Am Surg. 2013;79(11):1171-1176.
3. Lohani K, Shetty S, Sharma P, **Govindarajan V**, Thomas P, Loggie B. Pseudomyxoma Peritonei: Inflammatory Responses in the Peritoneal Microenvironment. Ann Surg Oncol. 2014; 21(5):1441-1447.
4. Shetty S., Bathla, L., **Govindarajan V.**, Thomas P. and Loggie B. W. Comparison of visceral sparing cytoreductive surgery and hyperthermic intraperitoneal chemotherapy with mitomycin or carboplatin for diffuse malignant peritoneal mesothelioma. *Am Surg* (2014); 80(4):348-352.
5. Foster JM, Radhakrishna U, **Govindarajan V**, Carreau JH, Gatalica Z, Sharma P, Nath SK, Loggie BW. Clinical implications of novel activating EGFR mutations in malignant peritoneal mesothelioma. World J Surg Oncol. 2010;8:88. PMCID: 2970593.

Additional recent publications

6. Kuracha MR, Siefker E, Licht JD, **Govindarajan V**. Spry1 and Spry2 are necessary for eyelid closure. Dev Biol. 2013;383(2):227-38. PMCID: 3857020.
7. Wolf L, Harrison W, Huang J, Xie Q, Xiao N, Sun J, Kong L, Lachke SA, Kuracha MR, **Govindarajan V**, Brindle PK, Ashery-Padan R, Beebe DC, Overbeek PA, Cvekl A. Histone posttranslational modifications and cell fate determination: lens induction requires the lysine acetyltransferases CBP and p300. Nucleic Acids Res. 2013;41(22):10199-214. PMCID: 3905850.
8. Kuracha MR, Burgess D, Siefker E, Cooper JT, Licht JD, Robinson ML, **Govindarajan V**. Spry1 and Spry2 are necessary for lens vesicle separation and corneal differentiation. Invest Ophthalmol Vis Sci. 2011;52(9):6887-97. PMCID: 3176024.
9. Rainey MA, George M, Ying G, Akakura R, Burgess DJ, Siefker E, Bargar T, Doglio L, Crawford SE, Todd GL, **Govindarajan V**, Hess RA, Band V, Naramura M, Band H. The endocytic recycling regulator EHD1 is essential for spermatogenesis and male fertility in mice. BMC Dev Biol. 2010;10:37. PMCID: 2856533.
10. Burgess D, Zhang Y, Siefker E, Vaca R, Kuracha MR, Reneker L, Overbeek PA, **Govindarajan V**. Activated Ras alters lens and corneal development through induction of distinct downstream targets. BMC Dev Biol. 2010;10:13. PMCID: 2828409.
11. Zhao M, Destache CJ, Zhan G, Liu H, Zhang Y, **Govindarajan V**, Opere CA. Regulation of retinal morphology and posterior segment amino acids by 8-isoprostaglandin E2 in bovine eyes ex vivo. Methods Find Exp Clin Pharmacol. 2008;30(8):615-26.
12. Zhang Y, Burgess D, Overbeek PA, **Govindarajan V**. Dominant inhibition of lens placode formation in mice. Dev Biol. 2008;323(1):53-63. PMCID: 2652847.
13. Zhang Y, Overbeek PA, **Govindarajan V**. Perinatal ablation of the mouse lens causes multiple anterior chamber defects. Mol Vis. 2007;13:2289-300.
14. **Govindarajan V**, Overbeek PA. FGF9 can induce endochondral ossification in cranial mesenchyme. BMC Dev Biol. 2006;6:7. PMCID: 1395304.
15. **Govindarajan V**, Harrison WR, Xiao N, Liang D, Overbeek PA. Intracorneal positioning of the lens in Pax6-GAL4/VP16 transgenic mice. Mol Vis. 2005;11:876-86.

D. RESEARCH SUPPORT:

Ongoing Research Support

NORD Govindarajan V (PI) 2/1/2015-1/31/2017
National Organization for Rare Disorders
Targeted parallel pathway blockade as a treatment option for pseudomyxoma peritonei (PMP)
The major goals of this proposal are a) to investigate the role of apoptotic protein BIM in mediating adaptive resistance to PI3K inhibition and b) to test the effectiveness of MEK and PI3K inhibitors in reducing tumor growth in patient-derived xenograft mouse models of PMP.
Role: co-investigator

NORD Govindarajan V (PI) 2/1/2014-1/31/2016
National Organization for Rare Disorders
PMP: Biologic foundations for new treatment options
The major goal of this proposal is to investigate the role of mutant KRAS in regulation of MUC2 expression in PMP
Role: PI

NORD Thomas P (PI) 2/1/2014-1/31/2016
National Organization for Rare Disorders
Carcinoembryonic Antigen a Pro-Angiogenic Factor in Pseudomyxoma Peritonei is a Potential Target for Therapy.
The major goal of this proposal is to study the role of CEA in angiogenesis in Pseudomyxoma Peritonei.
Role: co-investigator

Completed Research Support During the Last Three Years

2 P20 RR018788-06A1 Shelley Smith (PI) 9/15/2009-06/30/2014
NCRR
The Molecular Biology of Neurosensory Systems
This grant includes projects from six PIs from University of Nebraska Medical Center (UNMC), Boys Town and Creighton University that investigate cell cycle regulation, transcription factors, stem cells and microRNA mediated regulation of inner ear and retinal development.
Role: Mentor (this grant does not support my research)

George F. Haddix President's Faculty Research Fund Govindarajan V (PI) 2/1/2013-1/31/2014
The biological role of KRAS oncogene in pseudomyxoma peritonei
The major goal of this project is to define the role of mutant KRAS in MUC2 biosynthesis.
Role: PI

R01 EY017610-04 Govindarajan, V (PI) 9/01/2006-8/30/2012
NIH/NEI
Molecular Regulation of Ocular Gland Development
The major goals of this proposal are to analyze the roles of downstream components of the FGF-10 signaling pathway in regulation of lacrimal gland branching and differentiation.
Role: PI

Health Future Foundation Govindarajan, V (PI) 07/1/2011-6/30/2012
MicroRNA targets of the fibroblast growth factor signaling pathway
The goal of this project was to identify the microRNA targets of the FGF signaling pathway.
Role: PI

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Brian W. Loggie	Harold J. Bonnstetter Professor of Surgery Chief, Division of Surgical Oncology
eRA COMMONS USER NAME (credential, e.g., agency login)	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Loyola College, Montreal, Canada	CEGEP	1971-1973	Science
McGill University, Montreal, Canada	MD, CM	1973-1974	Pre-med
McGill University, Montreal, Canada	Internship	1974-1979	
Montreal General Hospital, Montreal, Canada	Residency	1979-1980	Surgery
Montreal General Hospital, Montreal, Canada	Clinical/Res.	1980-1986	Surgery
University of Illinois at Chicago, Chicago, Illinois	Fellowship	1986-1998	Surgical Oncology

A. Personal Statement

I am a practicing surgical oncologist with a busy national clinical practice with emphasis on peritoneal neoplastic disease, including appendix neoplasms, Pseudomyxoma Peritonei (PMP), Malignant Peritoneal Mesothelioma (MPM), Peritoneal Carcinomatosis, metastatic colorectal cancer (CRC), and ovarian cancer. We have an established a translational research program at Creighton University with infrastructure for obtaining blood and tissue from patients for these projects. In addition, virtually all patients have consented to be part of our research so that we can track clinical outcomes for correlative studies. We currently have a clinical database which continues to grow and is maintained on a regular basis. I have weekly research team meetings with the PI on this grant, Dr. Govindarajan, and other faculty and members of our Translational Research Working Group. This work has led to several articles that are either published, in press or in review (listed below under publications) and is the foundation for ongoing research. ***In addition, this research has altered my clinical practice.***

B. Positions and Honors.

PROFESSIONAL EXPERIENCE

1981-1983 Anatomy Demonstrator, Department of Anatomy, McGill University, Montreal, Canada
1986-1988 Clinical Instructor, Division of Surgical Oncology, University of Illinois at Chicago, Chicago, Illinois
1988-1995 Assistant Professor of Surgery, Division of Surgical Sciences, Bowman Gray School of Medicine, Winston-Salem, North Carolina
1994-2000 Cross-Appointment, Department of Cancer Biology, Bowman Gray School of Medicine, Winston-Salem, North Carolina
1995-2000 Associate Professor of Surgery, Division of Surgical sciences, Bowman Gray School of Medicine, Winston-Salem, North Carolina
2000-2002 Director, Surgical Oncology, UT Southwestern Moncrief Diagnostic Center, Fort Worth, Texas
2000-2002 Professor of Surgery, Division of Surgical Oncology, University of Texas Southwestern medical Center at Dallas, Dallas, Texas
2002- Director, Creighton Cancer Center, Creighton University School of Medicine, Omaha, Nebraska
2002- Professor of Surgery, Chief Division of Surgical Oncology, Creighton University School of Medicine, Omaha, Nebraska
2001-2014 Elected by his peers for inclusion in Best Doctors in America.
2007- Holder of the Dr. Harold J. Bonnstetter Endowed Chair Preventive Medicine, School of Medicine, Creighton University School of Medicine

C. Selected peer-reviewed publications (***from a total of 65 peer reviewed publications***) **(past three years)**

1. Kusamura S, Moran BJ, Sugarbaker PH, Levine EA, Elias D, Baratti D, et al. Multicentre study of the learning curve and surgical performance of cytoreductive surgery with intraperitoneal chemotherapy for pseudomyxoma peritonei. Br J Surg. 2014;101(13):1758-65.

2. Lohani K, Shetty S, Sharma P, Govindarajan V, Thomas P, **Loggie B.** Pseudomyxoma Peritonei: Inflammatory Responses in the Peritoneal Microenvironment. Ann Surg Oncol. 2014; 21(5):1441-1447.

3. Shetty S., Bathla, L., Govindarajan V., Thomas P. and **Loggie B.** Comparison of visceral sparing cytoreductive surgery and hyperthermic intraperitoneal chemotherapy with mitomycin or carboplatin for diffuse malignant peritoneal mesothelioma. Am Surg (2014); 80(4):348-352.

4. Levine EA, Stewart JHt, Shen P, Russell GB, **Loggie B**, Votanopoulos KI. Intraperitoneal Cytoreductive Surgery and Hyperthermic Intraperitoneal Chemotherapy for Peritoneal Surface Malignancy: Experience with 1,000 Patients, *Journal of the American College of Surgeons* (2014), 218(4):573-85.

5. Shetty S, Thomas P, Ramanan B, Sharma P, Govindarajan V, **Loggie B.** Kras mutations and p53 overexpression in pseudomyxoma peritonei: association with phenotype and prognosis. J Surg Res. 2013;180(1):97-103.
6. Shetty S, Natarajan B, Thomas P, Govindarajan V, Sharma P, **Loggie B.** Proposed classification of pseudomyxoma peritonei: influence of signet ring cells on survival. Am Surg. 2013;79(11):1171-6.

7. Foster JM, Gupta PK, Carreau JH, Grotz TE, Blas JV, Gatalica Z, Nath S, **Loggie B.** Right hemicolectomy is not routinely indicated in pseudomyxoma peritonei. Am Surg. 2012;78(2):171-7.

Selected peer-reviewed publications (older than three years)

8. Foster JM, Radhakrishna U, Govindarajan V, Carreau JH, Gatalica Z, Sharma P, Nath SK, **Loggie B**. Clinical implications of novel activating EGFR mutations in malignant peritoneal mesothelioma. World J Surg Oncol. 2010;8:88. PMCID: 2970593.
9. Helm, C Richard, S, Pan, J, Bartlett, D, Goodman, M, Hoefer, R, Lentz, S, Levine, E, **Loggie, B**, Metzinger, D, Miller, B, Parker, L, Spellman, J, Sugarbaker, P, Edwards, R, Rai, S. Hyperthermic Intraperitoneal Chemotherapy in Ovarian Cancer – First Report of the HYPER-O Registry. J Surg Oncol. 2010;20(1):61-69.

10. Gatalica Z, Foster JM, **Loggie B.** Low grade peritoneal mucinous carcinomatosis associated with human papilloma virus infection: case report. Croat Med J. 2008;49(5):669-73. PMCID: 2582360.

11. Levine EA, Stewart JHt, Russell GB, Geisinger KR, **Loggie B,** Shen P. Cytoreductive surgery and intraperitoneal hyperthermic chemotherapy for peritoneal surface malignancy: experience with 501 procedures. J Am Coll Surg. 2007;204(5):943-53; discussion 53-5.

12. McQuellon RP, **Loggie B**, Fleming RA, Russell GB, Lehman AB, Rambo TD. Quality of life after intraperitoneal hyperthermic chemotherapy (IPHC) for peritoneal carcinomatosis. Eur J Surg Onc 2001;27:65-73.

13. **Loggie B**, Fleming RA, McQuellon RP, Russell GB, Geisinger KR, Levine EA. Prospective trial for the treatment of malignant peritoneal mesothelioma. Am Surg 2001;67:999-1003.

14. Gatalica, Z. and **Loggie, B**. Cox-2 expression in pseudomyxoma peritonei. Cancer Letters, 244, pgs. 86-90. 2006.

15. **Loggie B**, Perini M, Fleming RA, Russell GB, Geisinger K. Treatment and prevention of malignant ascites associated with disseminated intraperitoneal malignancies by aggressive combined-modality therapy. Am Surg 1997;63:137-43.

D. RESEARCH SUPPORT:

Ongoing Research Support

NORD Govindarajan V (PI) 2/1/2015-1/31/2017
National Organization for Rare Disorders
Targeted parallel pathway blockade as a treatment option for pseudomyxoma peritonei (PMP)
The major goals of this proposal are a) to investigate the role of apoptotic protein BIM in mediating adaptive resistance to PI3K inhibition and b) to test the effectiveness of MEK and PI3K inhibitors in reducing tumor growth in patient-derived xenograft mouse models of PMP.
Role: co-investigator

NORD Govindarajan V (PI) 2/1/2014-1/31/2016
National Organization for Rare Disorders
PMP: Biologic foundations for new treatment options
The major goal of this proposal is to investigate the role of mutant KRAS in regulation of MUC2 expression in PMP
Role: co-investigator

NORD Thomas P (PI) 2/1/2014-1/31/2016
National Organization for Rare Disorders
CEA a pro-angiogenic factor in PMP is a target for therapy
The major goal of this proposal is to set up in vitro studies to examine the effects of CEA and cytokines on the growth of human umbilical vascular endothelial cells (HUVEC), and microvascular endothelial cells (MVEC).
Role: co-investigator

Completed Research Support

George F. Haddix President's Faculty Research Fund Govindarajan V (PI) 2/1/2013-1/31/2014
The biological role of KRAS oncogene in pseudomyxoma peritonei
The major goal of this project is to define the role of mutant KRAS in MUC2 biosynthesis.
Role: co-investigator

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors. Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Peter Thomas, Ph.D.	POSITION TITLE Professor of Surgery and Biomedical Sciences
eRA COMMONS USER NAME PETERTHOMAS	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Wales, Cardiff, UK	B.Sc.	1964-68	Biochemistry
University of Wales, Cardiff, UK	Ph.D.	1968-71	Biochemistry
Chester Beatty Research Institute, London, UK.	MRC Fellow	1971-72	Cancer Biology
Chester Beatty Research Institute, London, UK.	AK Fellow	1972-1979	Cancer Biology

A. Personal Statement

My laboratory is a part of an active translational research program investigating molecular signals and pathways that drive peritoneal neoplastic growth. A major interest of our group both clinically and from a basic science standpoint, is the study of Pseudomyxoma Peritonei (PMP), a cancer arising from mucinous neoplasms of the appendix that disseminates throughout the abdominal cavity. These cancers result in elevated levels of carcinoembryonic antigen (CEA) in both blood and abdominal fluid. This research project investigates the many roles of CEA in controlling the microenvironment of gastrointestinal derived peritoneal cancers. I have been involved in CEA related research since 1975. My research group was the first to show that CEA was metabolized by Kupffer cells and that the result of this metabolism was the production of pro-inflammatory cytokines. We showed that CEA therefore changed the tumor microenvironment such that it increased adhesion to the hepatic endothelium and also protected the tumor from hypoxia related cytotoxicity. We were also the first to identify and clone the CEA receptor (CEAR). The CEAR is also active in elicited peritoneal macrophages. I was also involved in first showing that the presence of CEA enhanced the metastatic potential of colorectal cancer cell lines to the liver. I have also experience in investigating CEA function in mouse models of colorectal cancers.

Over many years as a PI I have been involved in the use of nude mice to examine metastatic potential and treatments for colorectal cancers. I am therefore aware of problems that may arise in the models that Dr. Govindarajan is proposing to use for his studies and advise him on corrective actions to take. In addition to experience with in vivo models I also have thirty plus years investigating colorectal cancer behavior in vitro. These experiences were gained at the Institute of Cancer Research in London, Harvard Medical School, Boston University School of Medicine and now more recently at Creighton University. I have had a number of NIH grant awards (PO1, RO1, R21) in the field of colorectal cancer and have served on both NIH and DOD study sections. I hold the position of Professor of Surgery at Creighton University.

B. Positions and Honors.

Professional Experience

1979–1985 Senior Research Associate, Gastrointestinal Research Lab, Mallory Inst of Pathology, Boston City Hospital, Boston, Mass
1979–1982 Associate in Medicine, Harvard Medical School, Boston, Mass
1982–1985 Research Associate in Pathology, Scientific Staff, Boston City Hospitals, Boston, Mass
1982–1985 Principal Associate in Medicine, Harvard Medical School, Boston, Mass
1985–1988 Associate Member, Cancer Research Inst, New England Deaconess Hospital, Boston, Mass
1985–1990 Assistant Professor of Surgery (Biochemistry), Harvard Medical School, Boston, Mass
1988–1999 Senior Scientist, New England Deaconess Hospital, Boston, Mass

1990–1999 Associate Professor of Surgery (Biochemistry), Harvard Medical School, Boston, Mass
1999–2005 Professor of Surgery, Professor of Pathology and Laboratory Medicine, Director of Surgical Biology Labs, Boston University School of Medicine, Boston, Mass
2005- Professor of Surgery and Biomedical Sciences, Creighton University, Omaha NE.

Other Experience and Professional Memberships

1991 Reviewer for Queen Elizabeth II Research Fund, Canada
1992 Reviewer for Fonds FCAR, Quebec Canada
1995- Reviewer (Merit Grants) Department of Veterans Affairs
1996 Program Project Grant site visit team, Medical Research Council of Canada
1996 Program Project Grant site visit team, National Cancer Institute, NIH
1999–2000 Ad Hoc Member, MEP Study Section, NIH
1999–2003 Member ALTX-4 Study Section, NIH
2001-2005 Member USAMRMC Prostate Cancer Research Grant Review Panel (CET-3).
2003 ZRG1 ALTX-4, Reviewer for IRPG applications, NIH.
2003 External Reviewer for Canadian Institutes of Health Research.
2004 Reviewer Special Emphasis Panel/Initial Review Group ZRG1 DIG-B, (04) NIH.
2004 Reviewer Special Emphasis Panel/Initial Review Group 2004/05 ZRG1 DIG-F (02) NIH.
2003–2005 Scientist Reviewer, Breast Cancer Concept Awards, USAMRMC
2005-2008 Member Committee on Student Research and Scholarly Activities, Creighton University.
2007-2009 Member, the Graduate Program Coordinating Committee, Creighton University.
2008 Grant Reviewer; The Dutch Digestive Foundation.

Journal Referee

Reviewed manuscripts for: Cancer Research; New England Journal of Medicine; International Journal of Cancer; Cancer Letters, Clinical and Experimental Metastasis; Journal of Clinical Oncology; Gastroenterology; Hepatology, Canadian Journal of Physiology and Pharmacology; American Journal of Pathology

C. Selected Peer-Review Publications. (from 160 total peer reviewed publications)
Most relevant to the current application:

1. Zimmer R, **Thomas P**. Mutations in the carcinoembryonic antigen gene in colorectal cancer patients: Implications on liver metastasis. *Cancer Res*. 2001;61:2822-2826.
2. Bajenova OV, Zimmer R, Stolper E, Salisbury-Rowswell J, Nanji A, **Thomas P**. HnRNP M4 is a receptor for carcinoembryonic antigen in Kupffer cells. *J Biol Chem*, 2001;276:31067-31073.
3. Bajenova O, Stolper E, Gapon S, Sundina N, Zimmer R, **Thomas P**. Surface expression of HnRNP M4 nuclear protein on Kupffer cells relates to its function as a carcinoembryonic antigen receptor. *Exp Cell Res*. 2003;292:282-291.
4. Aarons, C.B., Bajenova, O., Andrews, C., Heydrick, S., Reed, K.L. **Thomas, P.,** Becker, J.M. and Stucchi, A.F. Carcinoembryonic antigen stimulated THP-1 macrophages activate endothelial cells and Increase cell-cell adhesion of colorectal cancer cells. *Clin. Exp. Metastasis* 2007, 24: 201-209,
5. **Thomas P,** Forse RA, Bajenova O, Carcinoembryonic antigen and its receptor hnRNP M are mediators of metastasis and the inflammatory response in the liver. *Clin Exp. Metastasis* 2011, 28: 923-932
6. Palermo, N.Y. **Thomas, P.** Murphy, R. J. and Lovas, S. Hexapeptide fragment of CEA that acts as antagonist of heterogeneous ribonucleoprotein M. *J. Peptide Science* 2012. 18:, 252-260.
7. Shetty S, Ramanan B, Sharma P, Govindarajan V, **Thomas P,** and Loggie B. Kras mutations and P53 overexpression in Pseudomyxoma Peritonei: Association with phenotype and prognosis. *J. Surg Res.* 2013 180(1):97-103
8. Shetty S, Natarajan B, **Thomas P.** Govindarajan V, Sharma P, and Loggie B. Proposed Classification of Pseudomyxoma peritonei. Influence of signet ring cells on survival. *The American Surgeon* 79(11):1171-610.
9. Shetty, S., Bathla, L., Govindarajan, V., **Thomas, P.** and Loggie, B. Comparison of visceral sparing cytoreductive surgery and hyperthermic intraperitoneal chemotherapy with mitomycin or carboplatin for diffuse malignant peritoneal mesothelioma. 2013 *American Surgeon* **80:** 348-352.

10. Lohani, K., Shetty, S., Sharma, P., Govindarajan, V., **Thomas, P.** and Loggie, B. Pseudomyxoma peritonei: inflammatory responses in the peritoneal microenvironment. 2013 *Ann Surg Oncol* **21:** 1441-1447

Additional publications (in chronological order):

11. **Thomas, P.,** Hayashi, H., Zimmer, R. and Forse, R. A. Regulation of cytokine production in carcinoembryonic antigen stimulated Kupffer cells by β2-adrenergic receptors. Cancer Letters. 2004; **209:** 251-257.
12. Jessup JM, Laguinge L, Lin S, Samara R, Aufman K, Battle P, Frantz M, Edmiston KH, **Thomas P**. Carcinoembryonic antigen induction of IL-10 and IL-6 inhibits hepatic ischemic/reperfusion injury to colorectal carcinoma cells. *Int J Cancer*. 2004;111:332-337.
13. Laguinge L, Bajenova O, Bowden E, Sayyah J, **Thomas P**, Jhul H. Nuclear protein hn RNP M4 interacts with CEA and might mediate signal transduction activity of CEA in HT29 colon cancer cells. *Anti Cancer Res*. 2005; 25: 23-32.
14. Bajenova, O.; Chaika; N. Tolkunova, E; Davydov-Sinitsyn A**; Thomas P**, O'Brien, S. Carcinoembryonic antigen and its binding partner hnRNPM, complex with adherens junction proteins. *Exper. Cell Res.* 2014 **324:** 115-123
15. McVicker, B. Tuma, D. Lazure KE, **Thomas P** and Casey C. Alcohol, Carcinoembryonic Antigen Processing and Colorectal Liver Metastases. In: *Advances in Experimental Medicine and Biology*, Springer, 2015; 815: 295-311.

D. RESEARCH SUPPORT

Ongoing Research Support

R21 Thomas P and Carol Casey (PIs) 10/1/2013-9/31/2015
National Institutes of Health
Alcohol altered CEA processing. Role in liver metasases in colorectal cancer.
The major goal of this grant is to determine the effects of alcohol on liver metastasis from colon cancers
Role: PI

NORD Thomas P (PI) 2/1/2014-1/31/2015
National Organization for Rare Disorders
Carcinoembryonic Antigen a Pro-Angiogenic Factor in Pseudomyxoma Peritonei is a Potential Target for Therapy.
The major goal of this grant is to study the role of CEA in angiogenesis in Pseudomyxoma Peritonei.
Role: PI

NORD Govindarajan V (PI) 2/1/2015-1/31/2017
National Organization for Rare Disorders
Targeted parallel pathway blockade as a treatment option for pseudomyxoma peritonei (PMP)
The major goals of this proposal are a) to investigate the role of apoptotic protein BIM in mediating adaptive resistance to PI3K inhibition and b) to test the effectiveness of MEK and PI3K inhibitors in reducing tumor growth in patient-derived xenograft mouse models of PMP.
Role: co-investigator

NORD Govindarajan V (PI) 2/1/2014-1/31/2015
National Organization for Rare Disorders
PMP: Biologic foundations for new treatment options
The major goal of this proposal is to investigate the role of mutant KRAS in regulation of MUC2 expression in PMP
Role: co-investigator

Completed Research Support

George F. Haddix President's Faculty Research Fund Govindarajan V (PI) 2/1/2013-1/31/2014
The biological role of KRAS oncogene in pseudomyxoma peritonei

The major goal of this project is to define the role of mutant KRAS in MUC2 biosynthesis.
Role: co-investigator

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
ALEKHA K. DASH	**PROFESSOR & CHAIR**
eRA COMMONS USER NAME (credential, e.g., agency login) AKDASH	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Jadavpur University, Calcutta, India	B. Pharm.	1981	Pharmacy
Jadavpur University, Calcutta, India	M. Pharm.	1983	Pharmacy
University of Minnesota, Minneapolis, Minnesota	Ph.D.	1990	Pharmaceutics

Please refer to the application instructions in order to complete sections A, B, C, and D of the Biographical Sketch.

A. Personal Statement: My Ph.D. research work involved the development and characterization of implantable polymeric drug delivery systems to treat bone infections. Our laboratory here at Creighton developed an *in situ* gel drug delivery system for the treatment of breast cancer (*DOD Concept Award; BC045664*'. The delivery system was so designed that when injected close to the site of the tumor, at the biological pH (7.4), the deprotonation of the ionic polymer portion in the delivery system turns it into an instant gel at the site of injection. This provided a sustained release of PTX from the *in situ* forming gel at and around the site of cancer while the systemic drug concentration was negligible. We have also been working on a nanoscale delivery system consisting of chitosan-GMO for the sustained local delivery of chemotherapeutics (LB692-State of Nebraska Grant). This patented delivery system has shown tremendous therapeutic effectiveness with reduced toxicity as compared to available commercial products. Recently we received an US Patent (**8,242,165**) for this innovative delivery. For the last several years our team at Creighton University was involved in developing a multifunctional nanodelivery system that includes chemotherapeutic agents and iron nanoparticle for hyperthermic treatment. A seed grant was awarded to our interdisciplinary collaborative team to generate some preliminary data for advancing this research. We are excited with the preliminary data, and hope this grant will provide financial support to address some of the unanswered questions, and fill the gap that still exists in the development of an optimal inhalable drug delivery system for lung cancer treatment. We have sufficient background and experiences to develop surface conjugated chitosan microparticulate inhalation delivery system for lung cancer treatment. Given my extensive experience and expertise in the area of drug delivery, I am in a strong position to advise Dr. Venkatesh Govindarajan (GV) and help him choose an appropriate dosing regimen for the PI3K small molecule inhibitor he is proposing to use on his mouse models.

B. Positions and Honors

1990-1995 Assistant Professor, Department of Pharmaceutical and Administrative Sciences, Creighton University, Omaha, Nebraska.
1994-present Adjunct Associate Professor, Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, Nebraska.
1995-2003 Associate Professor with Tenure, Department of Pharmacy Sciences, Creighton University, Omaha, Nebraska.
2003-present Professor, Department of Pharmacy Sciences, Creighton University, Omaha, Nebraska.
2004-2007 Interim Chair, Department of Pharmacy Sciences, Creighton University, Omaha, Nebraska.
2007-present Chair, Department of Pharmacy Sciences, Creighton University, Omaha, Nebraska.
2007-present Member of On-site Evaluation Team for Accreditation Council for Pharmacy Education.
2008-present Gilbet F. Taffe, Jr. Endowed Chair of Pharmacy Sciences, Creighton University, Omaha, Nebraska.
2009-2012 Reviewer for NIH Study Section: National Cancer Institute Special Emphasis Panel: Innovative Methods: Manufacturing Cancer Therapeutics (ZCA1 SRRB-D (C1).

Other Experience and Professional Memberships

1990-present	Member, American Association of Colleges of Pharmacy.
1991-present	Member, American Association of Pharmaceutical Scientists.
1994-present	Member, American Association for the Advancement of Science.
1995-1997	Chair and Organizer of the New Drug Delivery Systems Section of the Cells and Materials Meeting.
1995-present	Member of the Editorial Board, Analytical Profiles of Drug Substances and Excipients.
1995-present	Member, The New York Academy of Sciences.
1996	Chair and Organizer of the Drug Delivery Systems Section of the 1996 Fine Particle Society Meeting.
2003-2004	Chair, Preformulation Focus Group, American Association of Pharmaceutical Scientists.

Awards and Honors

1981	Following awards for securing the First Rank in the B. Pharm. program: IDMA G. P. Nair Gold Medal, University Medal and P. Das Memorial Award.
1983	The following Awards for securing the First Rank in the M. Pharm. program: University Medal and A. Sengupta Memorial Award.
1985-1988	Samuel Melendy Fellowship, University of Minnesota.
1986-present	Member of Rho Chi Honor Society.
1991 and 1993	Recipient of the John C. Kenefic Faculty Development Award, Creighton University.
1993	Outstanding Teacher of the Year: Nominated by the First Year Pharmacy Class
1993, 1999 and 2001	Recipient of the Pharmaceutical Research and Manufacturers Association Undergraduate Research Fellowship in Pharmaceutics.
1994-present	Member of Phi Beta Delta Honor Society for International Scholars.
1995, 2000, 2003	Who's Who Among Americas Teachers.
1995-present	Who's Who in the World, in America, and in Science and Engineering.
1996	Member Phi Lambda Sigma National Pharmacy Leadership Society.
1996, 1997	Certificate of Recognition for Excellence in Teaching, Department of Pharm. & Adm. Sci., Creighton University.
1996	Excellence in Teaching Award, Creighton University, School of Pharmacy and Allied Health Professions.
1997, 1998	Scholarly Achievement Award, Department of Pharm. & Adm. Sci., Creighton University.
1997	Visiting Professor, University of Technology, Jamaica, West Indies.
2000	Nominated for the AAPS Outstanding Educator Award.
2004 and 2005	Nominated for the Robert F. Kennedy Memorial Award for Teaching Achievement, Creighton University
2012	Elected as a **AAPS Fellow**

Patents

1. Hydroxyapatite based Drug Delivery Implant for Cancer Treatment, US Patent # 6767550, July, 2004.
2. Foam-film system with antibacterial Properties and Methods for Reducing Bacterial Contamination in Animal Carcasses, Filled on November 15, 2003. International Application #: PCT/US04/038094.
3. Mucoadhesive nanoparticles for cancer treatment, US Patent # 8,242,165, August 2012.
4. Trademark registration (Reg. No. 3,687,057) on “NANOME™”. Registered on Sept 22, 2009.

C. Selected Peer-reviewed Publications (*from a total of 60 peer reviewed publications*)

Most relevant to the current application

1. Dash, A. K. and Suryanarayanan, R., An Implantable Dosage Form for the Treatment of Bone Infections, Pharm. Res., 9 (1992) 993-1002.
2. Dash, A. K., and Cudworth, G., Therapeutic Applications of Implantable Drug Delivery Systems, J. Pharmacol. Toxico. Method , 40 (1)(1998) 1-12.
3. Fan, H. and Dash, A. K., Effect of Cross-linking on the In Vitro Release Kinetics of Doxorubicin from Gelatin Implants, Int. J. Pharm., 213 (2001) 103-116.
4. Ganguly, S. and Dash, A.K., A Novel In Situ Gel for Sustained Drug Delivery and Targeting, Int. J. Pharma. 276 (2004) 83-92.

5. Panyam, J., Williams, D., Dash, A.K., Leilie-Pelecky, D. and Labhasetwar, V., Solid-state Solubility Influences Encapsulation and Release of Hydrophobic Drugs from PLGA/PLA Nanoparticles, J. Pharm. Sci., 93 (2004) 804-1814.
6. Jauhari, S. and Dash, A.K., A Mucoadhesive In-Situ Gel Delivery System for Paclitaxel, AAPS PharmSciTech 2006; 7 (2) Article 53.
7. Trickler, W.J., Nagvekar, A.A., and Dash, A.K., A Novel Nanoparticle Formulation for Sustained Paclitaxel Delivery, AAPS PharmSciTech 2008 Mar. 18.
8. Nagvekar, A., Trickler, W. and Dash, A.K., Current Analytical Methods Used in the In Vitro Evaluation of Nano-Drug Delivery Systems, Current Pharmaceutical Analysis 5 (2009) 358-366..
9. Trickler, W., Nagvekar, A., Dash A.K., The In Vitro Sub-cellular Localization and In Vivo Efficacy of Novel Chitosan/GMO Nanostructure Containing Paclitaxel. Pharm. Res., 26 (2009) 1963-1973.
10. Nagvekar, A., Trickler, W. and Dash, A.K., Chitosan and Glyceryl Monooleate Nanostructures Containing Gemcitabine: Potential Delivery System for Pancreatic Cancer Treatment. AAPSPharmSciTec (March 18, 2010) DOI: 10.1208/s12249-010-9393-0.
11. Destache, C.J., Belgum, T., Christensen, K, Shibata, A, Sharma, A. and Dash, A.K., Combination Antiretroviral Drugs in PLGA Nanoparticle for HIV-1. BMC Infectious Diseases 2009, 9:198.
12. Singh, S. and Dash, A.K., Paclitaxel in cancer treatment: Perspectives and Prospects of its Delivery Challenges, *Critical Reviews in Therapeutic Drug Carrier Systems*. 26 (2009) 333-372**.**
13. Dash, A.K., The Dark Side of Paclitaxel, *Oncol Rev*, 4 (2010) 71-72.
14. Trickler, W., Munt, D.J, Jain, N., Dash A.K., Antitumor efficacy, Tumor Distribution and Blood Pharmacokinetics of Chitosan/Glyceryl –Monooleate Nanostructure Containing Paclitaxel. Nanomedicine 6 (3) (2011) 437-448.
15. Pandit A.A. and Dash A.K., Surface-modified Solid Lipid Nanoparticulate Formulation for Ifosfamide: Development and Characterization. Nanomedicine 6 (8) (2011) 1397-1412.

D. Research Support

Ongoing Research Support

NORD Govindarajan V (PI) 2/1/2015-1/31/2017
National Organization for Rare Disorders
Targeted parallel pathway blockade as a treatment option for pseudomyxoma peritonei (PMP)
The major goals of this proposal are a) to investigate the role of apoptotic protein BIM in mediating adaptive resistance to PI3K inhibition and b) to test the effectiveness of MEK and PI3K inhibitors in reducing tumor growth in patient-derived xenograft mouse models of PMP.
Role: co-investigator

NORD Govindarajan V (PI) 2/1/2014-1/31/2016
National Organization for Rare Disorders
PMP: Biologic foundations for new treatment options
The major goal of this proposal is to investigate the role of mutant KRAS in regulation of MUC2 expression in PMP
Role: co-investigator

Completed Research Support

President's Faculty Research Grant Dash, A.K (PI) and Hulce, M. (CO-PI) 3/1/2011- 2/28/2012.
Novel Skin-Bound Melanoidins-Sunscreen Delivery Approach to Melanoma
The objective is to develop a long acting sunscreen using melanoidins reactions on the skin stratum corneum for melanoma prevention.
Role: Investigator

LB 692 New Initiatives Research Grant Program Dash, A.K (PI) 7/1/2010-6/30/2011
Nanome™: A Novel Nanodelivery System for Tumor Targeting
The major objective is to evaluate the effectiveness of Nanome™ *in vivo*
Role: Investigator

Creighton
UNIVERSITY

Sponsored Programs Administration

July 8, 2015

Dr. Deniz Yilmazer-Hanke
Department of Biomedical Sciences
School of Medicine

Dear Dr. Yilmazer-Hanke:

Thank you for submitting an application to the Cancer and Smoking Disease Research Development Grant Program. We received many impressive and highly competitive proposals. I am pleased to inform you that your application was among the most outstanding applications, and has therefore been selected for funding. Your application committee composite score is 36 and reviewer comments are attached.

I congratulate you on this successful application and look forward to the progression of your research program as you seek to establish extramural funding for this exciting project.

This award will be from July 1, 2015 through June 30, 2016. Your proposal will be funded by the Creighton University LB-595 mechanism in the amount of $60,000. Funding for the second year of your project will be contingent on adequate progress during the initial year of the award.

Please submit a Creighton Budget form to Sponsored Programs Administration as soon as possible so that we can establish a fund number for this award.

I thank you for your time and interest in this program.

Sincerely,

Thomas F. Murray

Thomas F. Murray, Ph.D.
Associate Vice Provost for Research and Scholarship

Cc: Sponsored Programs Administration
Jerrod Lawrence

Location: Criss I, Room 108 Mail: 2500 California Plaza • Omaha, NE 68178
phone: 402.280.2064 • fax: 402.280.4766 • www.creighton.edu/Grants

Creighton University Internal Grant Application

Face Page

1. TITLE OF PROJECT *(Do not exceed 200 characters, including spaces and punctuation.)*
Regulation of ITIH3 by Nicotine and Tobacco Smoke Through the CD44 Receptor

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☐ NO ☒ YES
(If "Yes," Check appropriate box to identify the program to which you are submitting)

Check One

- ☐ **Health Science Strategic Investment Fund Faculty Development Grants**
- ☐ **LB692 - NE Tobacco Settlement Biomedical Research Development New Initiative Grant**
- ☒ **LB595 - Cancer and Smoking Disease Research Program Development Grant**

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR

3a. NAME (Last, first, middle)	3b. DEGREE(S)
Yilmazer-Hanke, Deniz	MD PhD

3c. POSITION TITLE	3d. MAILING ADDRESS *(Street, city, state, zip code)*
Associate Professor	Dept of Biomedical Sciences Creighton University Criss II, Rm 314B 2500 California Plaza Omaha, NE 68178, U.S.A.
3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT Biomedical Sciences	
3f. MAJOR SUBDIVISION	
3g. TELEPHONE AND FAX *(Area code, number and extension)* TEL: 402-280-2965 FAX: 402-280-2690	E-MAIL ADDRESS: denizyilmazer-hanke@creighton.edu

4. HUMAN SUBJECTS RESEARCH	4a. Research Exempt	If "Yes," Exemption No.
☒ No ☐ Yes	☐ No ☐ Yes	

4b. Federal-Wide Assurance No.	4c. Clinical Trial	4d. NIH-defined Phase III Clinical Trial
00001078	☐ No ☐ Yes	☐ No ☐ Yes

5. VERTEBRATE ANIMALS	5a. Animal Welfare Assurance No.
☐ No ☒ Yes	A3348-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs($)
07/01/2015	6/30/2017	60,000	60,000	120,000	120,000

9. APPLICANT ORGANIZATION	10. TYPE OF ORGANIZATION
Name: Creighton University Address: 2500 California Plaza Omaha, NE 68178	Public: → ☐ Federal ☐ State ☐ Local Private: → ☒ Private Nonprofit For-profit: → ☐ General ☐ Small Business ☐ Woman-owned ☐ Socially and Economically Disadvantaged
	11. ENTITY IDENTIFICATION NUMBER 1470376583A1
	DUNS NO. 05-330-9332 Cong. District NE-002

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE	13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Beth J. Herr	Name: Beth J. Herr
Title: Director, Sponsored Programs Administration	Title: Director, Sponsored Programs Administration
Address: Creighton University 2500 California Plaza Omaha, NE 68178	Address: Creighton University 2500 California Plaza Omaha, NE 68178
Tel:402-280-5769 FAX:402-280-4766	Tel: 402-280-5769 FAX: 402-280-4766
E-Mail: grantsadmin@creighton.edu	E-Mail: grantsadmin@creighotn.edu

Program Director/Principal Investigator (Last, First, Middle): Yilmazer-Hanke, Deniz

DETAILED BUDGET FOR INITIAL BUDGET PERIOD DIRECT COSTS ONLY	FROM	THROUGH
	July 1, 2015	June 30, 2016

List PERSONNEL *(Applicant organization only)*
Use Cal, Acad, or Summer to Enter Months Devoted to Project
Enter Dollar Amounts Requested *(omit cents)* for Salary Requested and Fringe Benefits

NAME	ROLE ON PROJECT	Cal. Mnths	Acad. Mnths	Summer Mnths	INST.BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Yilmazer-Hanke, Deniz	PD/PI							
To be named	Technician	12			32,000	32,000	9,248	41,248
SUBTOTALS →						32,000	9,248	41,248

CONSULTANT COSTS	
EQUIPMENT *(Itemize)*	
SUPPLIES *(Itemize by category)* Oxidative stress assays 1,500, Immunohistochemistry, Western blotting, in situ hybridization $ 4,000; qPCR/molecular $ 3,500; general lab costs $ 725	9,725
TRAVEL Networking, conferences, symposia, etc.	800
INPATIENT CARE COSTS	
OUTPATIENT CARE COSTS	
ALTERATIONS AND RENOVATIONS *(Itemize by category)*	
OTHER EXPENSES *(Itemize by category)* Animal purchasing and housing $ 7,727 Open access and page charges $ 500	8,227

CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*		**$** 60,000
CONSORTIUM/CONTRACTUAL COSTS	FACILITIES AND ADMINISTRATIVE COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		**$** 60,000

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS	INITIAL BUDGET PERIOD *(from Form Page 4)*	2nd ADDITIONAL YEAR OF SUPPORT REQUESTED	3rd ADDITIONAL YEAR OF SUPPORT REQUESTED	4th ADDITIONAL YEAR OF SUPPORT REQUESTED	5th ADDITIONAL YEAR OF SUPPORT REQUESTED
PERSONNEL: *Salary and fringe benefits. Applicant organization only*.	41,248	42,772			
CONSULTANT COSTS					
EQUIPMENT					
SUPPLIES	9,725	10,436			
TRAVEL	800	1,000			
INPATIENT CARE COSTS					
OUTPATIENT CARE COSTS					
ALTERATIONS AND RENOVATIONS					
OTHER EXPENSES	8,227	5,792			
DIRECT CONSORTIUM/ CONTRACTUAL COSTS					
SUBTOTAL DIRECT COSTS *(Sum = Item 8a, Face Page)*	60,000	60,000			
F&A CONSORTIUM/ CONTRACTUAL COSTS					
TOTAL DIRECT COSTS	60,000	60,000			

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD **$** 120,000

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

BUDGET JUSTIFICATION - DIRECT COSTS

A. Personnel Year 1- $ 41,248, Year 2 - 42,772

Yilmazer-Hanke, Deniz, PhD, Principal Investigator. The PI will be responsible for the successful execution of the project including directing and supervising the design and planning of experiments, requisite analyses, and publications. Dr. Yilmazer-Hanke will train the technician in standard laboratory safety and performing all of the necessary experimental bench work, and will supervise the work of staff on a continuous basis. The PI will always be available for discussing the significance and implications of the results obtained, and monitor the integrity of experimental data.

Research Technician, TBN, 12 calendar months (100 % effort). The technician will be responsible for performing all activities and experiments described in the project. He/she will maintain the CD44 knockout (ko) and ITIH3 ko mouse colonies including regular breeding, obtaining tissue for genotyping, weaning and keeping the records in compliance with IACUC protocols. The technician will further perform daily drug delivery (e.g., nicotine, ascorbic acid, vehicle) and exposure to smoking chamber. Other duties will include (i) collecting blood and tissue, (ii) carrying out genotyping (including DNA isolation/PCRs/gels), (iii) isolating RNA and running quantitative RT-PCR (qRT-PCR), (iv) performing oxidatve stress assays, protein isolation, Western blotting and ELISA, and (v) contributing to data analysis and publication of results.
Base Salary (for 12 calendar months): The base salary is $ 32,000 in year 1 with a 3 % increment in year 2.
Fringe Benefits: Benefits are $ 9,248 calculated at a rate of 28.90 % of base salary for 2015/16 and at a rate of 29.77 % of base salary for 2016/17.

B. Laboratory Supplies Year 1 - $ 9,725, Year 2 – 10,436

Oxidative stress assay: $ 1,500 per year
Oxidized (GSSG) and reduced (GSH) glutathione standards, glutathione reductase, and chemicals required for the reaction [e.g., 5-Sulfosalicylic acid dehydrate, NADPH, 5,5′-Dithiobis(2-nitrobenzoic acid)]

Western blots/ELISAs: $ 4,000 per year
Primary/secondary and capture/detection antibodies for hyaluronan (HA) and ITIH3, standards (HA oligomer, ITIH3 recombinant protein), avidin-HRP, TMB and chemiluminescence based ELISA detection solutions, Bradford solution, buffers, acrylamide gels, membranes, ELISA plates, etc.

Quantitative RT-PCR (qRT-PCR) and molecular biology costs: Year 1 - $ 3,500; Year 2 - $ 4,000
SYBR® Green PCR Master Mix, 2-Pack (2 x 5 mL) (400rxn), Ambion mirVana™ RNA Isolation Kit (40), Ambion Turbo DNA-free kit for DNA digestion (50), SuperScript® III Reverse Transcriptase (10,000 Units = 50 rxns), MicroAmp® Fast Optical 96Well Rxn Plt w/Barcode, 0.1mL, RNaseOUT™ Recombinant Ribonuclease Inhibitor.
PCRs supplies for genotyping (primers, Taq-polymerase, buffers, nucleotide mix, DNA ladders), and material for gel electrophoresis of DNA/RNA samples (agarose, formaldehyde, etc.).

General lab costs: Year 1 - $ 725; Year 2 – $ 936
Gloves, glassware, pipettes and their calibration, RNAse zap solution, RNAse/DNAse free tubes and pipette tips, gel chambers, parafilm, filters, supplies for buffers and running gels, waste disposal, liquid nitrogen, etc.

C. Animal purchasing and housing costs Year 1 - $ 7,727; Year 2 $ 4,992

Purchase of CD44 knockout (ko) and control mice: Year 1 - $ 2,735

CD44 knockout mice characterized as SJL.129-Cd44tm1Ugu/J mice (congenic; targeted null mutation) and the control strain (SJL/J) will be purchased from Jackson Laboratory. The costs for cryorecovery of the CD44 knockout mice are $2,525.00 (at least two mice that carry the mutation will be provided). From the control strain, 3 breeder pairs will be purchased ($29.35 per female and $27.35 per male at 4 wks of age). This gives total costs of $ 2,735 [=$2,525.00 for cryorecovery + 3* ($29.35 + $27.35) for background controls + $40 shipment].

Housing of CD44 breeders: Year 1 - $1,774; Year 2 - $788

CD44 ko and SJL/J mice (background strain) will be crossed, because only littermates will be used for all experiments. To maintain the colony and to obtain a sufficient number of animals for the experiments, 8-10 breeding pairs (on average 18 mice) per strain will be held in the first year. This gives $1,774 (=18 mice x 365 days/year x $0.27/mouse and day) for breeders.
In the second year, the number of breeders will be reduced to 4 pairs, giving $ 788 (=8 mice x 365 days/year x $0.27/mouse and day)

Housing of ITIH3 breeders: Year 1 - $788; Year 2 - $1,774

In the first year of the project, the number of breeders will be low, because mice will be held in quarantine. Altogether 4 pairs of mice will be held, giving $ 788 (=8 mice x 365 days/year x $0.27/mouse and day).
In the second year ITIH3 ko (ko-first) mice will be crossed with C57BL6/J mice (background strain of embryonic stem cells purchased). For experiments, littermates derived from these breeders will be used. To maintain the colony and to obtain a sufficient number of animals for the experiments, 8-10 breeding pairs (on average 18 mice) per strain will be held in the second year. This gives $1,774 (=18 mice x 365 days/year x $0.27/mouse and day) for breeders.

Experimental CD44 animals: Year 1 - $ 2,430

The expected ratio of genotypes are CD44-/- : CD44-/+ : CD44+/+ = 1:2:1. There are 3 genotypes (heterozygous mice for studying gene dosage effects) and 6 treatment types (no exposue + vehicle; nicotine + vehicle; tobacco smoke + vehicle; no exposure + ascorbic acid; nicotine + ascorbic acid; tobacco smoke + ascorbic acid). The number of experimental groups is 18 giving 180 mice for using 10 mice per group (= 3 genotypes x 6 treatments x 10 mice/group). Because mice are weaned at the age of 3 weeks and drug treatment is initiated at 6 wks of age, the experimental mice are held 3 wks until the experiments and another 4 wks for chronic drug treatment, giving 7 wks (~ 50 days). Thus, the total costs for holding experimental animals are: 180 mice x 50 days x $0.27/mouse and day = $ 2,430 for experimental animals.

Experimental ITIH3 animals: Year 2 - $ 2,430

The expected ratio of genotypes are ITIH3-/- : ITIH3-/+ : ITIH3+/+ = 1:2:1. There are 3 genotypes (heterozygous mice for studying gene dosage effects) and 6 treatment types (no exposue + vehicle; nicotine + vehicle; tobacco smoke + vehicle; no exposure + ascorbic acid; nicotine + ascorbic acid; tobacco smoke + ascorbic acid). The number of experimental groups is 18 giving 180 mice for using 10 mice per group (= 3 genotypes x 6 treatments x 10 mice/group). Because mice are weaned at the age of 3 weeks and drug treatment is initiated at 6 wks of age, the experimental mice are held 3 wks until the experiments and another 4 wks for chronic drug treatment, giving 7 wks (~ 50 days). Thus, the total costs for holding experimental animals are: 180 mice x 50 days x $0.27/mouse and day = $ 2,430 for experimental animals.

D. Other: Year 1 – $ 500, Year 2 – $ 800
Publication costs (page charges, color figures, open access, etc.) and other dissemination costs.

E. Travel: Year 1 – $ 800, Year 2 – $ 1,000
Costs budgeted for networking and attending national and international meetings, symposia and conferences.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Deniz Yilmazer-Hanke	Associate Professor in Neuroscience Dept of Biomedical Sciences, Creighton University, Criss II, Rm 314B, 2500 California Plaza Omaha, NE 68178, U.S.A. Tel: (1) 402-280-2965; Fax: (1) 402-280-2690
eRA COMMONS USER NAME (credential, e.g., agency login) MDY24369	

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of Magdeburg, Germany	PD Dr. habil.	06/2004	Amygdala behavior, neurodegeneration
Ludwig-Maximilians-University (LMU) Munich, Germany	PhD equivalent	04/1993	Olfactory axons during development
LMU University Munich, Germany	MD	05/1991	Medicine (Internship)

A. Personal Statement

The goal of my current investigations is to study the effect of nicotine and tobacco smoke on the regulation of ITIH3 through the CD44 receptor. Oxidative stress and hyaluronan depolymerization are suggested as major mecahnisms involved in the down regulation of ITIH3 by tobacco smoke, and potentially also by nicotine, the major component of tobacco smoke. The project focuses on effects of oxidative stress and hyaluronan depolymerization in various organs including the brain, and anti-oxidant treatment to reverse nicotine-/tobacco smoke-induced adverse effects. The current project builds on my previous work in mice generated in my laboratory, which show a role of ITIH3 in stress-related changes. The project will allow us to establish pharmacological tools to modulate ITIH3 expression and to analyze nicotine-induced oxidative stress and HA fragmentation for the regulation of stress-related behaviors in future projects. In the past, my work has been funded by the *German Research Foundation* (SFB 426, TP B4/B5; SFB TR3 TP B2/C3) and various sources in Ireland including the *Health Research Board Ireland*, *both of which are highly competitive research support at national level.* Most recently, I obtained funding in the US. I have trained. At present, 1 PhD student, and 4 undergraduate students are working in my laboratory. Thus, I was successful in recruiting funding in Germany, Ireland and the US for several projects, in which I supervised technicians, postdocs, and graduate and undergraduate students. I successfully administered those projects (e.g. staffing, research protections, budget), collaborated with other researchers, and produced several peer-reviewed publications from each project. My *h-index* has reached 18 (Jan 2014) showing that the quality of my work is recognized by my peers and researchers working in my field.

B. Positions and Honors

Positions and Employment

1993 – 1997 Research Assistant, Institute of Anatomy, University of Frankfurt (JWG), Germany
1997 – 2006 Senior Research Assistant, Institute of Anatomy, University of Magdeburg (OvG), Germany
2006 – 2011 College Lecturer, Department of Anatomy, University College Cork, Ireland
Oct 2011 – Associate Professor, Department of Biomedical Sciences, Creighton University, Omaha, NE

Professional activities, honors and awards

1998-	*Ad hoc* reviewer for journals –Brain Struct Funct, Fron Behav Neurosci, J Comp Neurol, Brit J Pharmacol, Behav Brain Res, Acta Neuropathol, Eur Neuropsychopharmacol, Brain Res, Cell Tissue Res, Neuropharmacology, Behav Brain Funct, J Neural Transm, J Negat Results Biomed, Anat Embryol, J Anat, Epilepsia, JAALAS
2005	Reviewer for EU-Commission, FP6-2005-LIFESCIHEALTH-6 call (Project type: STREPs)
2005	External examiner for PhD student
2006	DAAD (Deutscher Akademischer Austausch Dienst) travel award
2012	Health Future Foundation Faculty Development Award Creighton University
2012, 2014	Reviewer for Alzheimer's Association, International Research Grant Program
2014	Reviewer for the Medical Research Council U.K., Scheme Research Grant
2015	Reviewer for the European Science Foundation
Since 2014	Editorial Board Member of peer-reviewed journal Brain Structure and Function

Memberships

1994-	Anatomical Society - based in Germany (Anatomische Gesellschaft)
1994-	German Society for Neuropathology and Neuroanatomy (Deutsche Gesellschaft für Neuropathologie und Neuroanatomie, DGNN)
1998-	Society for Neuroscience
1998-	Federation of European Neuroscience Societies (FENS) and German Neuroscience Society (Neurowissenschaftliche Gesellschaft, NWG)

C. Selected Peer-reviewed Publications (Selected from 47 peer-reviewed publications; Corresponding author*)

Most relevant to the current application

1. **Yilmazer-Hanke D.M.***, Faber-Zuschratter H., Linke R., Schwegler H. (2002). Contribution of amygdala neurons containing peptides and calcium-binding proteins to fear-potentiated startle and exploration-related anxiety in inbred Roman high- and low-avoidance rats. Eur. J. Neurosci. 15:1206-1218.
2. **Yilmazer-Hanke D.M.***, Roskoden T., Zilles K., Schwegler H. (2003). Anxiety-related behavior and density of glutamate, GABAA, acetylcholine and serotonin receptors in the amygdala of seven inbred mouse strains. Behav. Brain Res. 145:145-159.
3. Reiser G.*, Striggow F., Hackmann C., Schwegler H., **Yilmazer-Hanke D.M.** (2004). Short-term down-regulation of the brain-specific, PtdIns(3,4,5)P3/ Ins(1,3,4,5)P4-binding, adapter protein, p42IP4 / centaurin-α1 in rat brain after acoustic and electric stimulation. Neurochem. Int. 45:89-93.
4. **Yilmazer-Hanke D.M.***, Hantsch M., Hanke J., Schulz C., Faber-Zuschratter H., Schwegler H. (2004). Neonatal thyroxine treatment: Changes in the number of corticotropin-releasing-factor (CRF) and neuropeptide Y (NPY) immunoreactive neurons and density of tyrosine hydroxylase positive fibers (TH) in the amygdala correlate with anxiety-related behavior of Wistar rats. Neuroscience 124:283-297.
5. Asan E.*, **Yilmazer-Hanke D.M.**, Eliava M., Hantsch M., Lesch K.-P., Schmitt A. (2005). The corticotropin-releasing factor (CRF)-system and monoaminergic afferents in the central amygdala: investigations in different mouse strains and comparison with the rat. Neuroscience 131:953-967.
6. Rose C., Röhl F.-W., Schwegler H., Hanke J., **Yilmazer-Hanke D.M.** * (2006). Maternal and genetic effects on anxiety-related behavior of C3H/HeJ, DBA/2J and NMRI mice in a motility-box following blastocyst transfer. Behav. Genet. 36:745-762.
7. D'Hanis W., Linke R., **Yilmazer-Hanke D.M.*** (2007). Topography of thalamic and parabrachial calcitonin gene-related peptide (CGRP) immunoreactive neurons projecting to subnuclei of the amygdala and extended amygdala. J. Comp. Neurol. 505:268-291.
8. Rose C., Röhl F.-W., Hanke J., Schwegler H., **Yilmazer-Hanke D.M.*** (2008). Maternal and genetic effects on the acoustic startle reflex and its sensitization in C3H/HeN, DBA/2J and NMRI mice following blastocyst transfer. Behav. Genet. 38:596-611. Farrelly L.A., Savage N.T.P., O'Callaghan C., Toulouse A., **Yilmazer-Hanke D.M.*** (2013) Therapeutic concentrations of valproate but not amitriptyline increase neuropeptide Y (NPY) expression in the human SH-SY5Y neuroblastoma cell line. Regul. Pept. 186:123-130.

9. Farrelly L.A., Savage N.T.P., O'Callaghan C., Toulouse A., **Yilmazer-Hanke D.M.*** (2013) Therapeutic concentrations of valproate but not amitriptyline increase neuropeptide Y (NPY) expression in the human SH-SY5Y neuroblastoma cell line. Regul. Pept. 186:123-130.
10. Browne C.A., Clarke G., Hanke J., Dinan T.G., Schwegler H., **Yilmazer-Hanke D.M.*,** Cryan J.F.* (2013) Alterations in prefrontal cortical serotonin and antidepressant-like behavior in a novel C3H/HeJxDBA/2J recombinant inbred mouse strain. Behav. Brain Res. 236:283-288.
11. O'Loughlin E.K., Pakan J.M.P., McDermott K.W., Deniz **Yilmazer-Hanke D. *** (2014) Expression of neuropeptide Y 1 receptors in the amygdala and hippocampus and anxiety-like behavior associated with Ammon's horn sclerosis following intrahippocampal kainate injection in C57BL/6J mice. Epilep. Behav. 37:175-183.
12. Browne C.A., Hanke J., Rose C., Walsh I., Foley T., Clarke G., Schwegler H., Cryan J.F., **Yilmazer-Hanke D.M.*** (2014) Effect of acute swim stress on plasma corticosterone and brain monoamine levels in bidirectionally selected DxH recombinant inbred mouse strains differing in fear recall and extinction. Stress 13:1-36.
13. Suryavanshi P.S., Ugale R.R, **Yilmazer-Hanke D.**, Stairs D.J., Dravid S.M.* (2014) GluN2C/GluN2D subunit-selective NMDA receptor potentiator CIQ reverses MK-801-induced impairment in prepulse inhibition and working memory in Y-maze test in mice. Brit. J. Pharmacol. 171:799–809.

Additional recent publications of importance to the field (Chronological order; *Corresponding author)

1. Hüttmann K., **Yilmazer-Hanke D**., Seifert G., Pape H.-C., Schramm J., Steinhäuser C.* (2006).Molecular and functional properties of neurons in the human lateral amygdala. Mol. Cell. Neurosci., 31:210-7.
2. **Yilmazer-Hanke D.M.***, Faber-Zuschratter H., Becker A., Blümcke I., Bickel M., Mawrin C., Schramm J. (2007) Axo-somatic inhibition of projection neurons in the lateral nucleus of amygdala in human temporal lobe epilepsy: An ultrastructural study. Exp. Brain Res. 177:384-399.
3. Janitzky K., Linke R., **Yilmazer-Hanke D.M.**, Grecksch G., Schwegler H.* (2007). Disrupted visceral feedback reduces locomotor activity and influences contextual fear conditioning in C57BL/6JOlaHsd mice. Behav. Brain Res. 182:109-118.
4. **Yilmazer-Hanke D.M.*** (2008) Morphological correlates of emotional and cognitive behaviour: Insights from studies on inbred and outbred rodent strains and their crosses. Behav Pharmacol. 19:403-34.
5. Frisch C.*, Hanke J., Kleinerüschkamp S., Thulke S., Richter S., Elger C.E., Schramm J., **Yilmazer-Hanke D.**, Helmstaedter C. (2009). Positive correlation between the density of neuropeptide Y-positive neurons in the amygdala and parameters of self-reported anxiety and depression in mesiotemporal lobe epilepsy patients. Biol. Psychiatry 66:433-440.
6. Faber-Zuschratter H., Hüttmann K., Steinhäuser C., Becker A., Schramm J., **Yilmazer-Hanke D.M.*** (2009) Ultrastructural characterisation of satellitosis in the human lateral amygdala associated with Ammon's horn sclerosis. Acta Neuropathol. (Berl.) 117:545–55.
7. Camp M.*, Norcross M., Whittle N., Feyder M., D'Hanis W., **Yilmazer-Hanke D.**, Singewald N., Holmes A. (2009). Impaired Pavlovian fear extinction is a common phenotype across genetic lineages of the 129 inbred mouse strain. Genes Brain Behav. 8:744-752.
8. Rose C., Schwegler H., Hanke J., **Yilmazer-Hanke D.M.*** (2012) Pregnancy rates, prenatal and postnatal survival of offspring, and litter sizes after reciprocal embryo transfer in DBA/2JHd, C3H/HeNCrl and NMRI mice. Therioneology 77:1883–93.
9. **Yilmazer-Hanke D.*** (2012). Amygdala. In: Paxinos G, Mai J.K. (eds) The Human Nervous System, 3rd Edition. Academic Press (Elsevier Ltd), London, pp.759-834.
10. . Manning J., Kulbida R., Rai R., Jensen L., Bouma J., Singh S.P., O'Malley D., **Yilmazer-Hanke D.*** (2014) Amitriptyline is efficacious in ameliorating muscle inflammation and depressive symptoms in the mdx mouse model of Duchenne muscular dystrophy. Exp. Physiol. 99:1370-1386.

D. Research Support

Ongoing Research Support

10/2011-9/2014 Nebraska LB692. Principal Investigator: **Yilmazer-Hanke D.** "Role of Emotional Changes in Limbic Areas and in Epilepsy." Equipment/Supplies/Technician. $ 300,000. *No cost extension until 6/2015*

2/2015 - 6/2015 NIH–NIGMS 8P20GM103471-09 (CoBRE). Principal Investigator: **Yilmazer-Hanke D.** "Generation of an ITIH3 knockout strain with knockout first mutation and conditional potential." $ 10,741.42

Pending Research Support

12/01/2015 - 11/30/2017 NIH R21 Resubmission. Principal Investigator: **Yilmazer-Hanke D.** Co-Investigator: Beisel K. "Effect of Inter-Alpha-Trypsin Inhibitor Heavy Chain 3 (ITIH3) on emotional learning in different genetic mouse models." Personnel, Supplies, Equipment, Travel, Publication costs: $ 275,000 plus indirect costs. *No overlap with current project.*

9/2015-9/2017 Brain Behavior Resarch Foundation (BBRF). Principal Investigator: **Yilmazer-Hanke D.** "Impact of blockade of mTOR pathway on the glucocorticoid-regulated gene Itih3 and exaggerated fear- and stress responses." PI salary (18 % effort), supplies travel, dissemination and 8% indirects, $ 100,000. - *No overlap with current project*.

7/2015 – 6/2016 State of Nebraska LB506. Principal Investigator: **Yilmazer-Hanke D.** "Regulation of ITIH3 by nicotine and the CD44 receptor." Cost share for PI salary (5 % effort), technician, supplies $50,000 direct costs. *There is overlap with Aim 1 of the current project.*

Completed Research Support (selected)

7/2012-6/2014 NIH–NIGMS 8P20GM103471-09 (Subaward 34-5507-2020-109 to **Yilmazer-Hanke D.M.**) "Cis-acting expression quantitative trait loci (eQTLs) in recombinant inbred lines differing in fear." Part-time postdoc, supplies: $ 80,000.- direct costs ($ 115,600 including indirects).

7/2012-6/2014 Health Future Foundation. Principal Investigator: **Yilmazer-Hanke D.M.** "miRNA differentially expressed in recombinant inbred lines differing in fear and effect of environmental enrichment." Supplies: $ 20,000.-

10/2010-9/2013 Health Research Board Ireland. Principal Investigator: **Yilmazer-Hanke D.M.** (until Oct 2011) **/ McDermott K.** "Expression of neuropeptide Y (NPY) and its receptors in the amygdala in human and experimental temporal lobe epilepsy. " 1 PhD student, consumables, travel: € 105,861.-

8/2010-7/2013 MeroPharm AG Principal Investigator: **Yilmazer-Hanke D.M.** "Role of glucose in altered prefrontal-limbic circuits on the development of streptozotocin-induced Alzheimer-like pathology in two different mouse models." Consumables, travel: € 18,000.-

12/2008-10/2011 Muscular Dystrophy Ireland. Principal Investigator: **Yilmazer-Hanke D.M.** Contribution of the inflammatory reaction in the muscle in Duchenne muscular dystrophy to depression: Development of new treatment strategies. MSc student, consumables, travel: € 37,616.-

4/2007-3/2008 MSc in Applied Biotechnology in UCC. Project leader: **Yilmazer-Hanke D.M.** "Induction of dysfunction in amygdala-prefrontal circuits in emotionally selected mice with different genetic backgrounds". € 5,640.- (fees of MSc student), consumables € 1,300.-

7/2004-6/2008 SFB /TR3, TP C3. Project leaders: Pape H.-C., **Yilmazer-Hanke D.M. (Co-PI)**, Steinhäuser C. "Functional and molecular properties of neurons in the human amygdala." *in Magdeburg (to D. Y.-H):*1 PhD student BAT-O IIa/2, consumables € 7,800.-/year plus € 3,500.-/year from the OvG Univ., renewal of a diamond knife € 1,000.- (2004), travel costs.

1/2003-12/2005 SFB 426, TP B4. Project leaders: Linke R., **Yilmazer-Hanke D.M. (Co-PI)** "Thalamo-amygdaloid information processing: modulation of anxiety and vegetative responses by amygdaloid peptides". 1 PhD student BAT-O IIa/2, consumables € 10,938/year, telemetry transmitters € 7,760 (2004), travel costs.

7/2001-6/2004 SFB /TR3, TP B2. Project leaders: **Yilmazer-Hanke D.M. (Lead-PI)**, Blümcke I., Schramm J., Schwegler H. "Epilepsy-related changes in the structural and molecular organisation of the human amygdala" In MD to D.Y.H.: 1 doctoral student BAT-O IIa/2, 1 technician BAT-O Vc/2, consumables € 7,670/year, Image-Analysis-System (2001) DM 26,000.-, travel costs.

RESEARCH PLAN

1. Specific Aims

Inter-Alpha-Trypsin Inhibitor Heavy Chain 3 (ITIH3) forms the heavy chain of a serine protease inhibitor. ITIH3 stabilizes the extracellular matrix by covalently binding hyaluronan (HA). HA polymers can scavenge reactive oxygen species resulting in HA depolymerization and the activation of the HA receptor CD44 by free HA fragments. Tobacco smoking induces oxidative stress, which mediates intracellular signaling cascades through HA fragments/CD44 as it was shown for activation of MAPK. In contrast, anti-oxidants prevent HA depolymerization and the effects mediated by free HA fragments. Thus, by binding to HA, ITIH3 may ameliorate tissue damaging effects of oxidative stress and reduce signaling through CD44 receptors.

Currently, there is a big trend for replacing tobacco-containing cigarettes with electronic nicotine delivery devices - better known as e-cigarettes - that deliver purified nicotine. However, the safety of pure nicotine delivery through e-cigarettes and the long-term effects are not known. **Smoking of tobacco-containing cigarettes down regulates ITIH3 in humans and also induces oxidative stress**. Currently, it is unclear whether the down regulation of ITIH3 is caused by nicotine itself or the by-products in tobacco-containing cigarettes. Furthermore, it is not known, through which mechanisms cigarette smoking down regulates ITIH3.

Because there is cumulating evidence that nicotine on its own can cause oxidative stress, we hypothesize that **nicotine treatment is sufficient to decrease ITIH3 expression by activating (i) the nicotinic and/or (ii) the CD44 receptor**, e.g., through depolymerization of HA by oxidative stress. The effects mediated by nicotinic receptors, tobacco smoke and the hyaluronan receptor CD44 will be investigated through chronic application of nicotine as well as by using CD44 knockout (ko) mice. In addition, the role of ITIH3 in nicotine-induced oxidative stress will be studied in ITIH3 ko mice.

Aim 1: Demonstrate that reduction of ITIH3 expression by nicotine- and/or smoking-induced oxidative stress is mediated by hyaluronan (HA) polymer fragments activating the CD44 receptor. ITIH3 expression and markers of oxidative stress (e.g., glutathione, lipid peroxidation) and HA fragmentation will be studied in CD44 ko mice and their controls (littermates) following chronic nicotine treatment or exposure to tobacco smoke. A chronic delivery mode has been chosen, because in the human population tobacco smoking or the use of e-cigarettes is often associated with chronic use. Levels of ITIH3 and oxidative stress/HA fragments will be examined in various organs including the lung, liver, kidney, urinary bladder, skeletal muscle and brain. It is hypothesized that nicotine/tobacco smoke will reduce ITIH3 levels in control mice, but fail to decrease ITIH3 in CD44 ko mice, because the HA fragments induced by oxidative stress will not be able to activate signaling cascades reducing ITIH3 expression. Thus, CD44 deficiency will protect from damage induced by HA fragments, because ITIH3 levels will remain high despite nicotine delivery, and by binding to HA polymers, ITIH3 will prevent HA fragmentation. Antioxidant delivery will reverse the nicotine-induced HA fragmentation and reduction of ITIH3.

Aim 2: Demonstrate that ITIH3 deficient mice have enhanced oxidative stress following nicotine treatment and/or exposure to tobacco smoke. The effect of a global deficit of ITIH3 on enhanced oxidative stress will be studied in ITIH3 ko mice. An ITIH3 ko mouse model with a ko-first design and conditional potential will be used, which will allow us to pursue a two-step approach. Homozygous/heterozygous Itih3 ko-first and controls littermates with a C57BL/6 background (background of embryonic stem cells used) will be studied for studying nicotine-induced HA fragmentation, glutathione levels and lipid peroxidation. The organs studied will include blood, lung, liver, kidney, urinary bladder, skeletal muscle and brain. Again, a chronic nicotine delivery scheme will be applied as explained above. The advantage of using ITIH3 ko-first mice is that ITIH3 conditional ko mice can be generated from the ITIH3 ko-first founder mice in follow-up projects, which will allow the use of a tamoxifen-driven cre-loxP-based system.

This approach will allow us to study mechanistic effects of nicotine-induced oxidative stress on ITIH3 expression. The results will show whether HA fragmentation and the CD44 receptor mediate the reduction of ITIH3 by nicotine and/or tobacco. If nicotine is sufficient to induce these changes, then antagonists against subtypes of nicotinic receptors can be used in future projects to identify the cellular pathways involved. Studies in ITIH3 ko mice will show whether ITIH3 ameliorates effects of nicotine-induced oxidative stress such as HA depolymerization. Furthermore, the ITIH3 ko-first mice used in the current project can serve as a founder strain for generating ITIH3 conditional ko mice for studying the role of ITIH3 expression under cell type-specific and temporal control.

2.Research Strategy

a. Significance: ITIH3 forms the heavy chain of a serine protease inhibitor involved in extracellular matrix stabilization, cell migration/attachment, and inflammation (Paris et al., 2002). It binds covalently HA to stabilize the extracellular matrix (Zhuo & Kimata, 2008; Zhang et al., 2012; Lauer et al., 2013). Therefore, ITIH3 may counteract the oxidative stress-induced degradation of HA and the resulting activation of CD44 signaling cascades (Casalino-Matsuda et al., 2009). ITIH3 may further improve the capacity of HA to scavenge reactive oxygen species by preventing its depolymerization. However, the protective effect of ITIH3 may be compromised by its down regulation through tobacco smoke, or potentially by the nicotine present in cigarettes, because reduced plasma ITIH3 levels were shown in individuals smoking tobacco-containing cigarettes (Bortner et al., 2011). Nevertheless, the latter study employed a screening approach based on proteomic analysis, and therefore did not provide information on whether tobacco smoking reduces ITIH3 synthesis or ITIH3 protein levels only, or whether nicotine, the major component in tobacco, is responsible for the reduction of ITIH3 in the plasma of smokers.

Nicotine is metabolized by liver enzymes into various metabolites such as cotinine, which can be further metabolized into pro-carcinogenic compounds, e.g., nicotine-derived nitrosamine ketones (Hecht et al., 1999; Benowitz, 2009). Both nicotine and its metabolites were made responsible for the induction of oxidative stress in various organs including the lung, kidney, pancreas, and the brain (Smith et al., 1995; Hecht et al., 1999; Kadlubar et al., 2009; Ande et al., 2012; Arany et al., 2013). Thus, nicotine delivery through e-cigarettes may be sufficient to induce oxidative stress-related tissue damage. Moreover, oxidative stress induced by tobacco smoke was shown to lead to fragmentation of HA polymers and the activation of the HA receptor CD44 by free HA fragments (Casalino-Matsuda et al., 2009; Monzon et al., 2010), although it remained unclear, whether these effects were mediated by nicotine or by other compounds in tobacco.

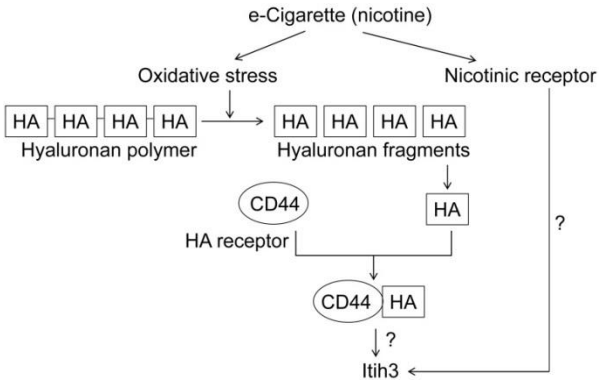

Figure 1: Tobacco smoking induces oxidative stress and down regulates ITIH3. Nicotine, the major component of tobacco and e-cigarettes, also causes oxidative stress. Because hyaluronan (HA) fragments induced by oxidative stress activate the HA-receptor CD44, it is hypothesized that nicotine down regulates ITIH3 through the activation of the CD44 receptor but not nicotinic receptors.

In the present project, the effect of nicotine administration on ITIH3 mRNA and protein levels will be investigated, because it is hypothesized that chronic nicotine treatment will reduce ITIH3 expression. Presence of HA fragmentation through nicotine-induced oxidative stress will also be confirmed. In addition, it is postulated that the activation of CD44 signaling by free HA fragments is responsible for the reduction in ITIH3 and this can be ameliorated with anti-oxidant treatment. The results of the project will provide insights into novel mechanism, through which nicotine can lead to tissue damage. The project outcome will also have a big impact on the delivery mode of nicotine during its use as a temporary measure to terminate tobacco smoking, e.g., by co-treatment with anti-oxidants to minimize adverse effects of nicotine. This approach will allow studying the mechanisms that regulate ITIH3 expression by chronic nicotine and/or tobacco smoke administration. We will identify whether the decrease in ITIH3 is a result of nicotine-induced oxidative stress and whether the activation of the CD44 receptor through HA fragments is responsible for the effects of nicotine. Moreover, the lack of protective effect in ITIH3 deficient mice will worsen the nicotine-induced oxidative stress and HA depolymerization.

b. Innovation

Tobacco smoke and its major component nicotine both can cause oxidative stress (Casalino-Matsuda et al., 2009; Arany et al., 2013). Therefore, the replacement of tobacco smoking by e-cigarettes, which contain pure nicotine, may not be safe, especially considering the long term use due to the addictive nature of nicotine and higher dosage of nicotine in e-cigarettes than in tobacco. Moreover, nicotine is often temporarily delivered in nicotine plasters to help quit smoking. ITIH3 levels are reduced in plasma of cigarette as smokers (Bortner et

al., 2010). ITIH3 is a HA-binding protein, which plays an important role in inflammation and oxidative stress-induced damage. Therefore, ITIH3 may be a new therapeutic target for the treatment of nicotine- and/or cigarette-induced oxidative stress. The experimental design will test whether nicotine administration on its own can induce HA fragmentation and down regulation of ITIH3. In addition, the potential role of tobacco by-products in reducing ITIH3 levels will be studied. Potential mechanisms of regulation of ITIH3 by nicotine and/or tobacco by-products through the CD44 receptor will be examined in CD44 and ITIH3 ko mice. In summary, the present proposal is aimed at investigating the hazards of nicotine itself, the mechanisms, through which nicotine exerts its effects, and a potential benefit of co-treatment with anti-oxidants during the clinical use of nicotine for quitting smoking.

c. Experimental approach

Aim 1. Show that hyaluronan (HA) depolymers resulting from oxidative stress following chronic nicotine treatment/tobacco smoke exposure decrease ITIH3 levels through the CD44 receptor. Not only tobacco, but nicotine and its metabolites also cause oxidative stress (Ande et al., 2012). Because oxidative stress leads to HA depolymerization through induction of the enzyme hyaluronidase (Monzon et al., 2010), the formation of HA fragments following nicotine and/or tobacco exposure should occur independently from the presence of the CD44 receptor. The impact of nicotine treatment on ITIH3 expression and the reversal of this nicotine effect with anti-oxidant treatment will be studied.

Objective: We will show that chronic nicotine and/or tobacco smoke delivery increases reactive oxygen species in CD44 and control mice. This will result in an increase in markers of oxidative stress and HA depolymerization. Furthermore, chronic nicotine delivery will decrease ITIH3 mRNA and/or protein levels in a CD44-dependent manner through binding of HA fragments to the CD44 receptor, and therefore the lack of the CD44 receptor will protect from ITIH3 down regulation. Anti-oxidant treatment will reverse oxidative stress, fragmentation of HA, and reduction of ITIH3 induced by nicotine/tobacco smoke.

Experimental design:

Subjects: CD44 ko mice (SJL.129-Cd44^{tm1Ugu}/J) will be purchased from Jackson Laboratory and bred in the animal housing of the PI's institution. Pups will be genotyped at the age of 10-14 d by obtaining tail tips. Young adult homozygous CD44 -/- heterozygous CD44 +/-, and homozygous control mice with CD44 +/+ alleles (littermates) will be used for the experiments.

Drug treatment: For nicotine treatment, mice will receive nicotine p.o. (200 µg/mL in drinking water; Arany et al., 2013), and control mice regular water. The effects of nicotine will be reversed with the anti-oxidant ascorbic acid (120 mg/kg i.p. dissolved in saline, adjusted to pH 7.35; Parle & Dhingra, 2003) and control mice will be administered the same volume of vehicle (saline i.p.).

Tobacco smoke exposure: Mice from the tobacco groups will be exposed to cigarette smoke for 3 hours per day for five days per week for a duration of 4 weeks (Ma et al., 2012). All other experimental groups will be handled like smoke-exposed animals except that they will be confined to chambers with room air for the period of smoke exposure. A TE-10 smoking device (Teague Enterprises, Davis, CA) will be used for controlled delivery of smoke (approximately 100 mg/m^3 TSP; TSP total suspended particle) (see Letter of Dr. Cullen). The mouse groups will be exposed to tobacco smoke for 6 hrs per day in custom built home cages allowing controlled delivery of smoke.

Sample collection: Tissue will be collected with the aim of obtaining at least 4 samples from each organ (trunk blood, skeletal muscle, lung, kidney, liver, urinary bladder, and brain). From bilateral organs, organs from both sides of the body (rt. right; lt. left) will be collected, and each organ will be divided into two halves as necessary (rt. and lt. rectus femoris muscle from leg versus rt. and lt. flexors of arm; superior plus and lobes versus inferior lobe of rt. and lt. lung; anterior and posterior halves of rt. and lt. kidney separated in parallel to the renal pelvis;). From the liver, the right, median and left lobes will be each divided into two halves). The urinary bladder will be divided into anterior and posterior sections, each of which will be halved. To study plasma, trunk blood will be collected in 1.5 ml EDTA-coated tubes, centrifuged for 15 min at 1000 g at 4ºC and the plasma obtained will be aliquoted (Manning et al., 2014). From the brain the rt. and lt. hippocampi, amygdalae, prefrontal cortices, striata and cerebella will be dissected out on an ice cold platform. Samples will be snap frozen using liquid nitrogen, and preserved at -80C until further use.

Oxidative stress/HA fragment measurement: Oxidative stress will be assessed using a glutathione assay by measuring total and oxidized glutathione levels (Sigma). Presence of HA oligomers will be shown using Western blotting, and HA oligomers will be quantified with a molecular weight/ELISA-like assay (Casalino-

Matsuda et al., 2006). Lipid peroxidation will be measured using a lipid hydroperoxide (LOOH) assay kit (Cayman Chemical Company, Ann Arbor, MI).

ITIH3 levels: For determining ITIH3 protein levels tissue from the other side of the body will be used. Tissue will be homogenized using RIPA buffer with protease inhibitors and protein will be isolated. Protein concentrations will be measured using a Bradford assay (Sigma) and a Western blot will be performed to show tissue expression pattern of ITIH3 protein using SDS-Page gel electrophoresis and semi-dry transfer. ITIH3 protein blotted on a polyvinylidene difluoride (PVDF) membrane will be detected with a primary (Santa Cruz) and secondary horseradish peroxidase-conjugated antibody and luminol reagent (Bio-Rad). Samples from tissues with confirmed ITIH3 expression and plasma will be analyzed using a commercial ELISA kit (Antibodies-online.com) to measure ITIH3 concentrations. For determining ITIH3 mRNA levels, total RNA will be isolated (mirVana kit, Ambion), which will be reverse transcribed into cDNA using random hexamer primers (Superscript III First Strand Synthesis System kit, Invitrogen Life Technologies). Quantitative RT-PCR will be carried out with optimized primers and SYBR®Green PCR Master Mix (Invitrogen Life Technologies) using a AB7500 Fast Real-Time PCR System (Applied Biosystems®). Succinate dehydrogenase complex, subunit A, flavoprotein (SDHA), which has been shown to be an effective control gene in qRT-PCR, will be used as a housekeeping gene (Farrelly et al., 2013).

Timeline of experiments: Delivery of nicotine, tobacco smoke and no exposure will be continued daily for 4 wks (28 days). In each group mice will also receive either ascorbic acid or vehicle (saline). Mice will be sacrificed on the 28th day of treatment (2hrs after last injection). Thus, it will be ensured that all mice are exposed to the smoke delivery device to have equal handling conditions in all groups.

Experimental animals: There are 3 different genotypes (CD44 -/-, +/-, +/+) and 6 treatment groups (no exposure+saline i.p., nicotine p.o.+saline i.p., tobacco smoke+saline i.p., no exposure+ascorbic acid i.p., nicotine p.o.+ascorbic acid, and tobacco smoke+ascorbic acid), which gives a total of 18 experimental groups. It is anticipated that 10 animals per group are sufficient to obtain statistically significant results (power of 0.80, two-tailed p-value <0.05), giving a total of 180 mice (18 groups x 10 mice/group = 180 mice). Data will be tested for normal distribution and analyzed with a two-way ANOVA (genotype x treatment) followed by a post hoc Tukey's test.

Expected Results, Interpretation, Possible Pitfalls: It is expected that the percentage of oxidized glutathione (GSSG) compared to total glutathione levels (GSH) and lipid peroxidation (LOOH assay) will be significantly elevated in nicotine- and tobacco smoke-treated mice from all groups (CD44 -/-, CD44 -/+ and wildtype (WT) controls) compared to the no exposure group. Likewise, the levels of HA oligomers will be increased following chronic nicotine treatment and tobacco exposure. Anti-oxidant treatment will reverse the oxidative stress-induced increase in GSSG and fragmentation of HA induced by nicotine and tobacco smoke. It is further hypothesized that HA fragments induced by nicotine delivery will down regulate ITIH3 expression through activation of the CD44 receptor. Therefore, nicotine and tobacco smoke will reduce ITIH3 mRNA and protein levels in WT mice, but not CD44 -/- mice due to the lack of activation of the CD44 receptor by HA fragments in the latter mice. Down regulation of ITIH3 may be also mediated direct nicotinic receptor activation and/or other signaling pathways (e.g., other HA receptors such as receptor for hyaluronan mediated motility RHAMM), which will be subject of future investigations. In this case, ITIH3 will not only be down regulated in WT mice, but also in CD44 -/- mice, especially after tobacco exposure shown to reduce ITIH3 levels. Moreover, CD44 deficiency may protect from nicotine- and/or tobacco smoke-induced inflammation without affecting ITIH3 levels, which will be analyzed to obtain supporting preliminary data for follow-up projects by studying cytokines and other inflammatory markers activated by HA fragments (e.g., in an R01 project). Finally, it is possible that the tobacco-related decrease in ITIH3 levels observed by Bortner and co-authors (2010) is induced by tobacco by-products rather than nicotine, which is assessed by the current experimental design. P.o. nicotine delivery is preferred due to the less invasive nature of this route of administration (e.g., minimizing inflammatory responses due to injections/surgery). Thus, low dose nicotine will be delivered through a per oral (p.o.) route, which was shown to cause oxidative stress in the kidney (Arany et al., 2013). Nevertheless, s.c. delivery of a higher nicotine dose (6.0 mg/kg/day s.c., Iwaniec et al., 2001) may be necessary for more effective nicotine treatment, which will be determined in preliminary experiments in this study (experiments will be initiated with 6-7 mice with p.o. low nicotine versus water treatment, and oxidative stress/HA fragmentation will be analyzed). Low dose p.o. nicotine treatment will be replaced by high dose nicotine delivery if necessary. An i.p. route of delivery is used for ascorbic acid to avoid delivering acidic drinking water and drug interactions in the water. In all experiments, littermates are studied to control genetic background effects.

Aim 2. Show that ITIH3 deficiency results in enhanced oxidative stress and hyaluronan (HA) depolymerization following chronic nicotine or tobacco smoke exposure. In this part of the project, we want to focus on the role of an ITIH3 deficit on nicotine- and/or tobacco smoke-induced oxidative stress and HA fragmentation in an ITIH3 ko mouse. This will be achieved by studying a newly generated ITIH3 ko mouse strain with a cassette containing a knockout-first design and conditional potential.

Objective: We will show that chronic nicotine and/or tobacco smoke delivery leads to a higher increase in reactive oxygen species in ITIH3 ko mice compared to wildtype mice. This will also lead to an enhanced depolymerization of HA. Anti-oxidant treatment will reverse oxidative stress and fragmentation of HA induced by nicotine and/or tobacco smoke.

Experimental design: Embryonic stem (ES) cell clones with an ITIH3 ko-first mutation (promoter driven cassette with reporter-tagged insertion and conditional potential) will be injected into mouse blastocysts (Fig.2). From the resulting chimeras, ITIH3 ko-first founder mice with constructs in the germ cell line will be obtained. Mice from these ITIH3 ko-first founder mice will be treated with nicotine, tobacco smoke or no exposure and tested for oxidative stress/HA fragmentation (see Aim 1). ITIH3 ko-first mice will be tail-clipped and genotyped at the age of 10-14d. Young adult homozygous ITIH3 -/- heterozygous ITIH3 +/-, and homozygous control mice with ITIH3 +/+ alleles (littermates) will receive these treatments for 4 wks (28 days). In addition, the effect of ascorbic acid will be tested. On day 28 day, the mice will be sacrificed and trunk blood and tissue from skeletal muscle, lung, kidney, liver, urinary bladder and brain will be collected as described above (see Aim 1). Samples will be snap frozen using liquid nitrogen, and preserved at -80C until further use. Oxidative stress will be assessed using a glutathione assay by measuring total and oxidized glutathione levels (Sigma) and by analyzing lipid peroxidation (LOOH assay, Cayman Chemical Company). HA oligomers will be detected in Western blots and quantified with a molecular weight/ELISA-like assay.

Timeline of experiments: Tissue will be collected on the 28th day of nicotine/tobacco/no exposure-treatment (2hrs after last injection). Groups will also receive ascorbic acid or saline and all mice will be exposed to the smoke delivery device to ensure equal handling conditions.

Experimental groups: Homozygous (-/-) and heterozygous (-/+) ITIH3 ko-first and wildtype (+/+) littermates will be used. In addition, there are 6 treatment groups (no exposure+saline, nicotine +saline, tobacco smoke+saline, no exposure+ascorbic acid, nicotine+ascorbic acid, and tobacco smoke+ascorbic acid). Total number of mice used is 180, as there are 18 (= 3 x 6) groups and experiments will be initiated with 10 animals per group (18 groups x 10 mice/group = 180 mice). Data will be tested for normal distribution and analyzed with a two-way ANOVA (genotype x treatment) followed by a post hoc Tukey's test. Animal numbers will be reduced if smaller group sizes provide statistically significant results (power of 0.80, two-tailed p-value <0.05)

Expected Results, Interpretation, Possible Pitfalls: It is expected that the percentage of oxidized glutathione (GSSG) compared to total glutathione levels (GSH) and lipid peroxidation ((LOOH assay) will be significantly higher in nicotine- and/or tobacco smoke-treated mice ITIH3 ko mice than in wildtype (WT) controls. Likewise, the levels of HA oligomers will be increased following chronic nicotine and/or tobacco smoke treatment. Moreover, anti-oxidant treatment will reverse the oxidative stress-induced increase in GSSG, lipid peroxidation and fragmentation of HA induced by nicotine and/or tobacco. ITIH3 ko mice are currently being generated in the mouse engineering core facility at UNMC with an estimated delivery by the end of 2015 (see funding from CoBRE). Therefore, experiments under Aim 2 are planned for year 2 of the project. During generation of ITIH3 ko mice, clone expansion will be performed in 2-3 reprogrammed (dedifferentiated) ES clones to improve success rate of the procedure (C56BL/6 background). ES cell injection into blastocyst will be repeated with new clones if germ line transmission fails. If heterozygous ITIH3 ko-first mutations prove lethal; the FRT-flanked stop cassette will be removed from ES cell lines themselves, and ES cells with the new construct (floxed Itih3 allele with conditional potential) will be injected into mouse blastocyst. Cryptogenic activity of ITIH3 may occur in ko mice, however, 3 transcripts are predicted to produce a truncated protein product, out of which 2 are subject to non-sense mediated decay, and a frame shift is expected following deletion of the critical exons 3-4 of the ITIH3 gene. We studied the presence of all three splice variants of ITIH3 with PCR in wildtype mice, which will be also monitored in ITIH3 ko mice. In Western blots, only one protein variant at 100kDa was detected in wildtype mice by using antibodies against both C- and N-termini. Also, the ITIH3 gene is located closely to ITIH1/4 on the same chromosome, but ITIH4 is approximately 10-12 kb away from ITIH3 and the exons 3-4 deleted are about 20 kb away from ITIH4. We will monitor potential alterations in ITIH1/4 levels with qPCR to exclude that the insertion of the loxP site/large cassette in the genomic locus (e.g., into intron) hasn't disrupted unknown regulatory sequences.

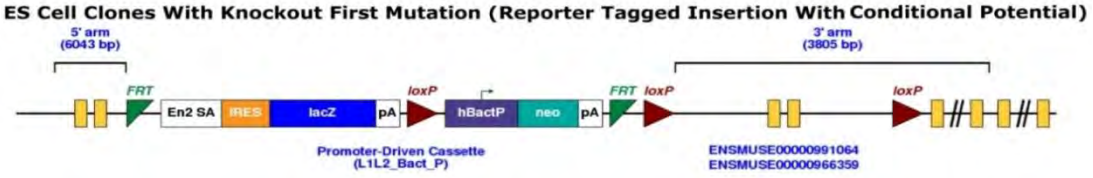

Fig.2: Construct with knockout first allele (promoter-driven selection cassette) used to generate the *Itih3* conditional ko mouse strain.

Cortical/Amygdalar Projection Neuron	Cortical/Amygdalar Interneuron	Glial cells
Camk2a-CreERT2	Gad2-CreERT2	GFAP-CreERT2
	Sst-CreERT2 (mostly interneurons)	Plp1-CreERT
	Pvalb-2A-CreERT2	
	nNOS-CreERT2	

Table 1: Examples of available strains with tamoxifen-inducible cre driven by cell-type specific promoters for generating ITIH3 conditional mice to study ITIH3 deficiency in the brain in follow-up projects.

Future directions: The project will allow the development of a new line of investigations. Smoking is a risk factor for premature birth and behavioral deficits in infants born to mothers that smoke. Therefore, in future projects the impact of ITIH3 and its regulation by the CD44 pathway on behavioral deficits will be analyzed. Furthermore, the newly generated ITIH3 mice have a knockfirst (ko-first) mutation with conditional potential. Therefore, these mice can be used to generate ITIH3 conditional ko mice by crossing the Itih3 ko-first founder mice with mice containing the Flp-Frt (flippase recombinase-flippase recombinase target) system to generate the floxed allele. For obtaining ITIH3 conditional ko mice, this floxed strain will be crossed with a tamoxifen-inducible CreER(T2) transgenic line with cre driven by cell specific promoters (Table 1, Jackson laboratory), so that loxP-flanked gene segment is excised in a tissue-/cell-type specific manner through Cre-mediated recombination upon tamoxifen administration. The distribution pattern of ITIH3 mRNA (Allen brain atlas) indicates neuronal Itih3 synthesis, but for selecting the appropriate tamoxifen-inducible CreER(T2) transgenic line, we will also characterize ITIH3 expressing cells by combining situ hybridization with double labeling for neuronal/glial markers. Cre-recombinase activity will be induced by tamoxifen delivery in the ITIH3 conditional ko strain, and experiments will be performed two weeks later (to allow degradation of proteins synthesized). Cell-type specific ITIH3 ko will be monitored with in situ hybridization combined with double labeling for respective markers. In summary, the outcome of the project will provide the basis for studying CD44 signaling pathways activated by nicotine, tobacco smoke and HA fragmentation for regulating the function of ITIH3 and other molecular partners. The data obtained will be used to support an R01 application to N.I.H.

Creighton University

February 6, 2015

Dear Committees Members:

In the proposed project, Dr. Deniz Yilmazer-Hanke wants to study the impact of nicotine treatment on the regulation of the ITIH3 gene via CD44 receptors. In the literature, it has been shown that cigarette smokers have reduced ITIH3 levels in plasma, but it is not known whether nicotine or tobacco-by-products are the cause. Therefore, Dr. Yilmazer-Hanke wants to add experimental groups treated with tobacco smoke to the study, if nicotine on its own does not induce a reduction in ITIH3 levels.

In my laboratory, I have a TE-10 smoking device (Teague Enterprises, Davis, CA) with chambers allowing controlled delivery of mainstream and sidestream tobacco smoke to rodents.

I will make the smoking device available to Dr. Yilmazer-Hanke throughout the experiments and wish her the best of luck for this interesting project.

Sincerely,

Diane M. Cullen

Diane M. Cullen, Ph.D.
Professor Biomedical Sciences

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Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

A grid of previous submissions and awards for the State LB506 program is included below.

Analysis of Submissions and Awards for the State of Nebraska LB 506 Funding		
Fiscal Year	**Submissions**	**Awards**
FY 03/04	**4**	**4**
FY 04/05	**0**	**0**
FY 05/06	**6**	**1**
FY 06/07	**11**	**2**
FY 07/08	**7**	**1**
FY 08/09	**9**	**3**
FY 09/10	**14**	**4**
FY 10/11	**7**	**4**
FY 11/12	**11**	**1**
FY 12/13	**5**	**0**
FY 13/14	**4**	**2**
FY 14/15	**1**	**1**
FY 15/16	**7**	**0**

Creighton University
Cancer & Smoking Disease Research Program
Total External Submissions & Awards

Investigator	Submitted FY 14/15	# Submitted
Thomas Murray	$1,492,469	3
Henry Lynch	$1,524,614	9
Laura Hansen	$1,991,801	4
Yaping Tu	$2,371,052	6
Patrick Swanson	$1,578,646	6
Xian-Ming Chen	$2,487,277	3
Peter Oldenburg	$237,575	1
Peter Abel	$0	0
Amy Arouni	$8,041	2
TOTAL SUBMISSIONS	$11,691,475	

Investigator	Awarded FY 14/15	# Awarded
Thomas Murray	$358,945	2
Henry Lynch	$103,107	2
Laura Hansen	$5,868	1
Yaping Tu	$588,614	3
Patrick Swanson	$100,950	1
Xian-Ming Chen	$694,638	2
Peter Oldenburg	$230,448	1
Peter Abel	$0	0
Amy Arouni	$8,041	2
TOTAL AWARDS	$2,090,611	

Sponsored Programs Administration

School of Medicine

From 7/1/2014 to 6/30/2015

PI/Agency	Directs	Indirects	Total	Project Title
Department: Pharmacology				
Awarded				
Murray, Thomas Prim Recp: University of California San Diego National Institutes of Health Dates: 07/01/12 - 06/30/15 Fund #: 278595-823800	$175,152	$77,942	$253,094	NEUROTOXINS FROM MARINE ALGAE AND CYANOBACTERIAL
Murray, Thomas Prim Recp: University of Kansas Center for Research, Inc. National Institutes of Health Sponsor #: FY2014-078 Dates: 09/01/13 - 08/31/18 Fund #: 278660-823800	$72,750	$33,101	$105,851	PEPTIDIC KAPPA OPOID RECEPTORS LIGANDS AS POTENTIAL TREATMENTS FOR DRUG ADDICTION
Total Awarded:	**$247,902**	**$111,043**	**$358,945**	
Submitted				
Murray, Thomas Prim Recp: University of California San Diego National Institutes of Health Sponsor #: 2R01NS053398-13 Dates: 07/01/12 - 06/30/15 Fund #: 278595-823800	$175,152	$77,942	$253,094	NEUROTOXINS FROM MARINE ALGAE AND CYANOBACTERIAL
Murray, Thomas Prim Recp: University of Florida U.S. Department of Defense Dates: 09/30/15 - 09/29/18	$226,803	$103,197	$330,000	NOVEL PEPTIDE ANTAGONISTS AS TREATMENTS FOR SUBSTANCE ABUSE
Murray, Thomas Prim Recp: University of Utah National Institutes of Health Dates: 09/01/15 - 08/31/20	$625,000	$284,375	$909,375	SYNTHESIS AND STUDY OF SMALL MOLECULE NEURONAL GROWTH PROMOTERS
Total Submitted:	**$1,026,955**	**$465,514**	**$1,492,469**	
School Awarded:	**$247,902**	**$111,043**	**$358,945**	**Total Awards: 2**
School Submitted:	**$1,026,955**	**$465,514**	**$1,492,469**	**Total Submissions: 3**

Sponsored Programs Administration

School of Medicine

From 7/1/2014 to 6/30/2015

Department: Preventive Medicine

Awarded

PI/Agency	Directs	Indirects	Total	Project Title
Lynch, Henry T Prim Recp: Weill Cornell College of Medicine National Institutes of Health Dates: 09/01/12 - 08/31/15 Fund #: 278618-825200	$41,251	$18,356	$59,607	WHOLE GENOME SEQUENCING TO DISCOVER FAMILIAL MYELOMA RISK GENES
Lynch, Henry T Prim Recp: University of Nebraska Medical Center National Institutes of Health Dates: 09/01/13 - 08/31/15 Fund #: 278674-825200	$29,897	$13,603	$43,500	PDQ3 GENETIC BASIS OF BREAST CANCER RESISTANCE IN BRCA1+CARRIER
Total Awarded:	**$71,148**	**$31,959**	**$103,107**	

Submitted

PI/Agency	Directs	Indirects	Total	Project Title
Lynch, Henry T Prim Recp: Weill Cornell College of Medicine National Institutes of Health Sponsor #: 12091283-01 R01CA167824-01A1 Dates: 09/01/12 - 08/31/15 Fund #: 278618-825200	$41,251	$18,356	$59,607	WHOLE GENOME SEQUENCING TO DISCOVER FAMILIAL MYELOMA RISK GENES
Lynch, Henry T Prim Recp: University of Alabama National Institutes of Health Dates: 07/01/15 - 06/30/19	$75,336	$33,460	$108,796	IDENTIFICATION OF GENETIC FACTORS FOR FAMILIAL LYMPHOID CANCERS
Lynch, Henry T Prim Recp: University of Nebraska Medical Center National Institutes of Health Sponsor #: 34-5150-2054-001 Dates: 09/01/13 - 08/31/15 Fund #: 278674-825200	$29,897	$13,603	$43,500	PDQ3 GENETIC BASIS OF BREAST CANCER RESISTANCE IN BRCA1+CARRIER
Lynch, Henry T Prim Recp: University of Arizona National Institutes of Health Dates: 07/01/15 - 06/30/20	$291,911	$132,820	$424,731	GENETIC PREDISPOSITION IN BRCAX FAMILIAL BREAST CANCER
Lynch, Henry T U.S. Department of Defense Dates: 09/01/15 - 08/31/18	$61,855	$28,145	$90,000	GERMLINE GENOME INSTABILITY CAUSED BY BRCA1+ PREDISPOSITION
Lynch, Henry T Prim Recp: Stanford University National Institutes of Health Dates: 07/01/15 - 06/30/20	$204,710	$93,145	$297,855	EVOLUTION AND SOMATIC LANDSCAPE OF LYNCH SNYDROME-ASSOCIATED TUMORS
Lynch, Henry T Prim Recp: University of Nebraska Medical Center National Institutes of Health Dates: 12/01/15 - 11/30/17	$27,492	$12,508	$40,000	PREDISPOSITION IN XRCC5 PROMOTER CONTRIBUTING TO FAMILIAL BREAST CANCER
Lynch, Henry T Prim Recp: University of Nebraska Medical Center National Institutes of Health Dates: 12/01/15 - 11/30/17	$41,237	$18,763	$60,000	EARLY GENOME INSTABILITY IN BRCA1+ FAMILIAL BREAST CANCER
Lynch, Henry T National Institutes of Health Dates: 12/01/15 - 11/30/17	$275,000	$125,125	$400,125	PREDICTIVE TESTING AND SCREENING UPTAKE IN LYNCH SYNDROME: ELECTRONIC EDUCATION VS. USUAL CARE
Total Submitted:	**$1,048,689**	**$475,925**	**$1,524,614**	

Sponsored Programs Administration

School of Medicine

From 7/1/2014 to 6/30/2015

PI/Agency	Directs	Indirects	Total	Project Title
School Awarded:	**$71,148**	**$31,959**	**$103,107**	**Total Awards: 2**
School Submitted:	**$1,048,689**	**$475,925**	**$1,524,614**	**Total Submissions: 9**

Sponsored Programs Administration

School of Medicine

From 7/1/2014 to 6/30/2015

PI/Agency	Directs	Indirects	Total	Project Title
Department: Biomedical Sciences				
Awarded				
Hansen, Laura Protransit Nanotechnology Dates: 12/01/14 - 11/30/15 Fund #: 212039-822380	$4,694	$1,174	$5,868	PROTECTIVE EFFICACY OF PLGA NANOPARTICLES ENCAPSULATING ANTIOXIDANT ENZYMES AGAINST RADIATION EXPOSURE
Total Awarded:	**$4,694**	**$1,174**	**$5,868**	
Submitted				
Hansen, Laura Protransit Nanotechnology Dates: 12/01/14 - 11/30/15 Fund #: 212039-822380	$4,694	$1,174	$5,868	PROTECTIVE EFFICACY OF PLGA NANOPARTICLES ENCAPSULATING ANTIOXIDANT ENZYMES AGAINST RADIATION EXPOSURE
Hansen, Laura State of NE-LB506 Dates: 07/01/15 - 06/30/16 **Closed: 05/31/15**	$50,000	$0	$50,000	ESTROGEN RECEPTOR ALPHA AND SQUAMOUS CELL CARCINOMA OF THE SKIN
Hansen, Laura National Institutes of Health Dates: 12/01/15 - 11/30/20	$1,284,275	$554,135	$1,838,410	MECHANISMS OF UV-INDUCED SKIN CARCINOGENESIS
Hansen, Laura Prim Recp: Protransit Nanotherapy National Institutes of Health Dates: 09/01/15 - 08/31/16	$69,229	$28,294	$97,523	DEVELOPING PRO-NP FOR SKIN CANCER PREVENTION
Total Submitted:	**$1,408,198**	**$583,603**	**$1,991,801**	
School Awarded:	**$4,694**	**$1,174**	**$5,868**	**Total Awards: 1**
School Submitted:	**$1,408,198**	**$583,603**	**$1,991,801**	**Total Submissions: 4**

Sponsored Programs Administration

School of Medicine

From 7/1/2014 to 6/30/2015

PI/Agency	Directs	Indirects	Total	Project Title
Department: Pharmacology				
Awarded				
Tu, Yaping U.S. Department of Defense Dates: 07/01/13 - 06/30/16 Fund #: 286023-823720	$125,000	$55,321	$180,321	ABERRANTLY UPREGULATED P-REX 1 PROMOTES CASTRATION-RESISTANT PROSTATE CANCER PROGRESSION
Tu, Yaping National Institutes of Health Dates: 08/01/13 - 04/30/17 Fund #: 270721-823720	$247,774	$110,519	$358,293	DYSREGULATION OF RGS2 PROTEIN AND AIRWAY HYPERRESPONSIVENESS IN ASTHMA
Tu, Yaping University of Nebraska Medical Center Dates: 11/01/14 - 10/31/15 Fund #: 288593-823720	$50,000	$0	$50,000	UP-REGULATED ANDROGEN RECEPTOR PROMOTES TRIAL RESISTANCE IN BREAST CANCER
Total Awarded:	**$422,774**	**$165,840**	**$588,614**	
Submitted				
Tu, Yaping U.S. Department of Defense Sponsor #: W81XWH-13-1-007 Dates: 07/01/13 - 06/30/16 Fund #: 286023-823720	$125,000	$55,321	$180,321	ABERRANTLY UPREGULATED P-REX 1 PROMOTES CASTRATION-RESISTANT PROSTATE CANCER PROGRESSION
Tu, Yaping National Institutes of Health Sponsor #: 1R01HL116849-03 Dates: 08/01/13 - 04/30/17 Fund #: 270721-823720	$497,774	$224,269	$722,043	DYSREGULATION OF RGS2 PROTEIN AND AIRWAY HYPERRESPONSIVENESS IN ASTHMA
Tu, Yaping University of Nebraska Medical Center Dates: 11/01/14 - 10/31/15 Fund #: 288593-823720	$50,000	$0	$50,000	UP-REGULATED ANDROGEN RECEPTOR PROMOTES TRIAL RESISTANCE IN BREAST CANCER
Tu, Yaping U.S. Department of Defense Sponsor #: BC150205 Dates: 01/01/16 - 12/31/18	$375,000	$170,625	$545,625	TARGETING ANDROGEN RECEPTOR AND TRAIL: A NOVEL TREATMENT PARADIGM FOR BREAST CANCER
Tu, Yaping Prim Recp: Clark Atlanta University National Institutes of Health Dates: 04/01/16 - 03/31/21	$312,500	$142,188	$454,688	TARGETING GIA2 IN METASTATIC PROSTATE CANCER
Tu, Yaping National Institutes of Health Sponsor #: 1R21CA193271-01A1 Dates: 04/01/16 - 03/31/18	$344,438	$73,937	$418,375	ANDROGEN RECEPTOR: A KEY REGULATOR OF TRAIL RESISTANCE IN BREAST CANCER?
Total Submitted:	**$1,704,712**	**$666,340**	**$2,371,052**	
School Awarded:	**$422,774**	**$165,840**	**$588,614**	**Total Awards: 3**
School Submitted:	**$1,704,712**	**$666,340**	**$2,371,052**	**Total Submissions: 6**

Sponsored Programs Administration

School of Medicine

From 7/1/2014 to 6/30/2015

PI/Agency	Directs	Indirects	Total	Project Title
Department: Medical Microbiology & Immunology				
Awarded				
Swanson, Patrick National Institutes of Health Dates: 07/01/15 - 06/30/16	$100,950	$0	$100,950	A FLOW CYTOMETER FOR MULTI-COLOR CELL ANALYSIS IN A MULTI-USER FACILITY
Total Awarded:	**$100,950**	**$0**	**$100,950**	
Submitted				
Swanson, Patrick National Institutes of Health Sponsor #: 5R01GM102487-04 Dates: 07/01/12 - 06/30/16 Fund #: 270709-824602	$202,696	$73,685	$276,381	ROLE OF VPRBP IN B CELL DEVELOPMENT AND V(D)J RECOMBINATION
Swanson, Patrick Prim Recp: University of Nebraska Medical Center National Institutes of Health Dates: 07/01/14 - 08/13/15 **Closed: 08/31/14**	$50,000	$0	$50,000	UNMC COBRE: DETRIMENTS OF THE B10 PHENOTYPE IN CHRONIC LYMPHOCYTIC LEUKEMIA
Swanson, Patrick National Institutes of Health Sponsor #: 1R21AI119829-01 Dates: 07/01/15 - 06/30/17 **Closed: 02/09/15**	$275,000	$125,125	$400,125	IMPLICATIONS OF B10-LIKE CELL EXPANSION IN A MODEL OF IMPAIRED RECEPTOR EDITING
Swanson, Patrick National Institutes of Health Sponsor #: 3R01GM102487-03S1 Dates: 07/01/15 - 06/30/16	$100,950	$0	$100,950	A FLOW CYTOMETER FOR MULTI-COLOR CELL ANALYSIS IN A MULTI-USER FACILITY
Swanson, Patrick Prim Recp: University of Nebraska Medical Center National Institutes of Health Dates: 09/01/15 - 08/31/16	$50,000	$0	$50,000	DETERMINANTS OF THE B10 PHENOTYPE IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)
Swanson, Patrick National Institutes of Health Dates: 02/01/16 - 03/31/17	$701,190	$0	$701,190	FACSARIA FUSION FLOW CYTOMETER
Total Submitted:	**$1,379,836**	**$198,810**	**$1,578,646**	
School Awarded:	**$100,950**	**$0**	**$100,950**	**Total Awards: 1**
School Submitted:	**$1,379,836**	**$198,810**	**$1,578,646**	**Total Submissions: 6**

Sponsored Programs Administration

School of Medicine

From 7/1/2014 to 6/30/2015

PI/Agency	Directs	Indirects	Total	Project Title
Department: Medical Microbiology & Immunology				
Awarded				
Chen, Xian-Ming National Institutes of Health Dates: 07/01/11 - 06/30/16 Fund #: 270691-824603	$198,624	$88,388	$287,012	EPITHELIAL EXOSOMES AND TLR-MEDIATED MUCOSAL DEFENSE
Chen, Xian-Ming National Institutes of Health Dates: 02/04/15 - 01/31/20 Fund #: 270727-824603	$280,155	$127,471	$407,626	MOLECULAR BASIS OF INTESTINAL CRYPTOSPORIDIOSIS
Total Awarded:	**$478,779**	**$215,859**	**$694,638**	
Submitted				
Chen, Xian-Ming National Institutes of Health Sponsor #: 1U01AI095532-05 Dates: 07/01/11 - 06/30/16 Fund #: 270691-824603	$195,680	$87,078	$282,758	EPITHELIAL EXOSOMES AND TLR-MEDIATED MUCOSAL DEFENSE
Chen, Xian-Ming National Institutes of Health Sponsor #: 1R01AI116323-01A1 Dates: 02/04/15 - 01/31/20 Fund #: 270727-824603	$1,458,632	$663,679	$2,122,311	MOLECULAR BASIS OF INTESTINAL CRYPTOSPORIDIOSIS
Chen, Xian-Ming Prim Recp: Oregon Health & Science University National Institutes of Health Dates: 07/01/15 - 06/30/16	$56,500	$25,708	$82,208	EXOSOMAL RNA SHUTTLING IN GI MUCOSAL IMMUNITY TO CRYPTOSPORIDIAL INFECTION
Total Submitted:	**$1,710,812**	**$776,465**	**$2,487,277**	
School Awarded:	**$478,779**	**$215,859**	**$694,638**	**Total Awards: 2**
School Submitted:	**$1,710,812**	**$776,465**	**$2,487,277**	**Total Submissions: 3**

Sponsored Programs Administration

School of Medicine

From 7/1/2014 to 6/30/2015

PI/Agency	Directs	Indirects	Total	Project Title
Department: Pharmacology				
Awarded				
Oldenburg, Peter National Institutes of Health Dates: 04/01/13 - 03/31/16 Fund #: 270716-823225	$159,480	$70,968	$230,448	ALCOHOL MODIFIES AIRWAY HYPERRESPONSIVENESS & INFLAMMATION IN ALLERGIC ASTHMA
Total Awarded:	**$159,480**	**$70,968**	**$230,448**	
Submitted				
Oldenburg, Peter National Institutes of Health Sponsor #: 4R00AA019499-05 Dates: 04/01/13 - 03/31/16 Fund #: 270716-823225	$164,412	$73,163	$237,575	ALCOHOL MODIFIES AIRWAY HYPERRESPONSIVENESS & INFLAMMATION IN ALLERGIC ASTHMA
Total Submitted:	**$164,412**	**$73,163**	**$237,575**	
School Awarded:	**$159,480**	**$70,968**	**$230,448**	**Total Awards: 1**
School Submitted:	**$164,412**	**$73,163**	**$237,575**	**Total Submissions: 1**

Sponsored Programs Administration

School of Medicine

From 7/1/2014 to 6/30/2015

PI/Agency	Directs	Indirects	Total	Project Title
Department: Medicine				
Awarded				
Arouni, Amy Janssen Scientific Affairs, L.L.C. Dates: 12/01/10 Fund #: 211744-848500	$2,897	$724	$3,621	OUTCOMES REGISTRY FOR BETTER INFORMED TREATMENT OF ATRIAL FIBRILLATION
Arouni, Amy Boehringer Ingelheim Pharmaceuticals, Inc. Dates: 09/01/12 Fund #: 211744-848500	$3,636	$784	$4,420	PROSPECTIVE, OPEN LABEL STUDY EVALUATING THE EFFICACY OF TWO MANAGEMENT STRATEGIES (PANTOPRAZOLE 40 MG Q.A.M.) AND TAKING PRADAXA WITH FOOD (WITHIN 30 MINUTES AFTER A MEAL) ON GASTROINTESTINAL SYMPTOMS (GIS) IN PATIENTS NEWLY ON TREATMENT WITH PRADAXA 150 MG B.I.D. OR 75 MG B.I.D. FOR THE PREVENTION OF STROKE AND SYSTEMIC EMBOLISM IN PATIENTS WITH NON-VALVULAR ATRIAL FIBRILLATION (NVAF)
Total Awarded:	**$6,532**	**$1,508**	**$8,041**	
Submitted				
Arouni, Amy Janssen Scientific Affairs, L.L.C. Sponsor #: RIVAROXAFL4001 Dates: 12/01/10 Fund #: 211744-848500	$2,897	$724	$3,621	OUTCOMES REGISTRY FOR BETTER INFORMED TREATMENT OF ATRIAL FIBRILLATION
Arouni, Amy Boehringer Ingelheim Pharmaceuticals, Inc. Sponsor #: 1160.128 Dates: 09/01/12 Fund #: 211744-848500	$3,636	$784	$4,420	PROSPECTIVE, OPEN LABEL STUDY EVALUATING THE EFFICACY OF TWO MANAGEMENT STRATEGIES (PANTOPRAZOLE 40 MG Q.A.M.) AND TAKING PRADAXA WITH FOOD (WITHIN 30 MINUTES AFTER A MEAL) ON GASTROINTESTINAL SYMPTOMS (GIS) IN PATIENTS NEWLY ON TREATMENT WITH PRADAXA 150 MG B.I.D. OR 75 MG B.I.D. FOR THE PREVENTION OF STROKE AND SYSTEMIC EMBOLISM IN PATIENTS WITH NON-VALVULAR ATRIAL FIBRILLATION (NVAF)
Total Submitted:	**$6,532**	**$1,508**	**$8,041**	
School Awarded:	**$6,532**	**$1,508**	**$8,041**	**Total Awards: 2**
School Submitted:	**$6,532**	**$1,508**	**$8,041**	**Total Submissions: 2**

Creighton University Cancer and Smoking Disease Research Program
LB595, Year 21 Progress Report (July 1, 2014 – June 30, 2015)

PUBLICATIONS
Thomas F. Murray, PhD, Principal Investigator

Discovery of Novel Mutations in Hereditary Cancer Program

Publications for Projects 1, 2 and 3 (Lynch):
[Note: An asterisk is placed at the beginning of each publication or grant directly related to LB595, followed by the related component number. Although Component I affects nearly every study we are involved in, we have designated as "directly related" some in which the data base updating has been especially instrumental.]

1. *I and III. Wen H, Kim YC, Snyder C, Xiao F, Fleissner EA, Becirovic D, Luo J, Downs B, Sherman S, Cowan KH, Lynch HT, Wang SM. Family-specific, novel, deleterious germline variants provide a rich resource to identify genetic predispositions for BRCAx familial breast cancer. *BMC Cancer* 2014;14:470.

2. *I. Park DJ, Tao K, Le Calvez-Kelm F, Nguyen-Dumont T, Robinot N, Hammet F, Odefrey F, Tsimiklis H, Teo ZL, Thingholm LB, Young EL, Voegele C, Lonie A, Pope BJ, Roane TC, Bell R, Hu H, Shankaracharya, Huff CD, Ellis J, Li J, Makunin IV, John EM, Andrulis IL, Terry MB, Daly M, Buys SS, Snyder C, Lynch HT, Devilee P, Giles GG, Hopper JL, Feng BJ, Lesueur F, Tavtigian SV, Southey MC, Goldgar DE. Rare mutations in RINT1 predispose carriers to breast and Lynch syndrome-spectrum cancers. *Cancer Discov* 2014;4:804-815.

3. *I. Giannakeas V, Lubinski J, Gronwald J, Moller P, Armel S, Lynch HT, Foulkes WD, Kim-Sing C, Singer C, Neuhausen SL, Friedman E, Tung N, Senter L, Sun P, Narod SA. Mammography screening and the risk of breast cancer in BRCA1 and BRCA2 mutation carriers: a prospective study. *Breast Cancer Res Treat* 2014;147:113-118.

4. Lynch HT, Drescher K, Knezetic J, Lanspa S. Genetics, biomarkers, hereditary cancer syndrome diagnosis, heterogeneity and treatment: a review. *Curr Treat Options Oncol* 2014;15:429-442.

5. Schlussel AT, Gagliano RA Jr, Seto-Donlon S, Eggerding F, Donlon T, Berenberg J, Lynch HT. The evolution of colorectal cancer genetics-Part 1: from discovery to practice. *J Gastrointest Oncol* 2014;5:326-335.

6. Schlussel AT, Gagliano RA Jr, Seto-Donlon S, Eggerding F, Donlon T, Berenberg J, Lynch HT. The evolution of colorectal cancer genetics-Part 2: clinical implications and applications. *J Gastrointest Oncol* 2014;5:336-344.

7. *I. Chai X, Friebel TM, singer CF, Evans DG, Lynch HT, Isaacs C, Garber JE, Neuhausen SL, Matloff E, Eeles R, Tung N, Weitzel JN, Couch FJ, Hulick PJ, Ganz PA, Daly MB, Olopade OI, Tomlinson G, Blum JL, Domchek SM, Chen J, Rebbeck TR. Use of risk-reducing surgeries in a prospective cohort of 1,499 BRCA1 and BRCA2 mutation carriers. *Breast Cancer Res Treat* 2014;148:397-406.

8. Gatalica Z, Snyder C, Maney T, Ghazalpour A, Holterman DA, Xiao N, Overberg P, Rose I, Basu GD, Vranic S, Lynch HT, Von Hoff DD, Hamid O. Programmed cell death 1 (PD-1) and its ligand (PD-L1) in common cancers and their correlation with molecular cancer type. *Cancer Epidemiol Biomarkers Prev* 2014;23:2965-2970.

9. Lanspa SJ, Lynch HT. Sessile serrated adenomas: why conventional endoscopy is okay for unconventional polyps. *Dig Dis Sci* 2014;59:2848-2849.

10. *III. Lynch H, Snyder C, Wang SM. Considerations for comprehensive assessment of genetic predisposition in familial breast cancer. *Breast J* 2015;21:67-75.

11. Bikhchandani J, Lynch HT. Commentary on 'Colonoscopy screening compliance and outcomes in patients with Lynch syndrome'. *Colorectal Dis* 2015;17:46-49.

12. Eskander RN, Lynch HT, Brown SM, Wagman LD, Tewari KS. Novel MSH2 mutation in the first report of a Vietnamese-American kindred with Lynch syndrome. *Gynecol Oncol Rep* 2015;12:31-33.

13. Lynch HT, Snyder CL, Shaw TG, Heinen CD, Hitchins MP. Milestones of Lynch syndrome: 1895-2015. *Nat Rev Cancer* 2015;15:181-194.

14. Hansford S, Kaurah P, Li-chang H, Woo M, Senz J, Pinheiro H, Schrader KA, Schaeffer DF, Shumansky K, Zogopoulos G, Santos TA, Claro I, Carvalho J, Nielsen C, Padilla S, Lum A, Talhouk A, Baker-Lange K, Richardson S, Lewis I, Lindor NM, Pennell E, MacMillan A, Fernandez B, keller G, Lynch H, Shah sP, Guilford P, Gallinger S, Corso G, Roviello F, Caldas C, Oliveira C, Pharoah PD, Huntsman DG. Hereditary diffuse gastric cancer syndrome: CDH1 mutations and beyond. *JAMA Oncol* 2015;1:306-313.

15. *I. Snyder C, Metcalfe K, Sopik V, Royer R, Zhang S, Narod SA, Akbari MR, Lynch HT. Prevalence of PALB2 mutations in the Creighton University Breast Cancer Family Registry. *Breast Cancer Res Treat* 2015;150:637-641.

16. Lynch HT, Lanspa SJ, Snyder CL, Drescher KM. Microsatellite instability, disease-free survival and role of tumour infiltrating lymphocytes (Invited editorial on 'Predictors of disease-free survival in colorectal cancer with microsatellite instability: An AGEO multicenter study'). *Eur J Cancer* 2015;51:922-924.

17. *I and III. Downs B, Kim YC, Xiao F, Snyder C, Chen P, Fleissner EA, Becirovic D, Wen H, Sherman S, Cowan KH, Lynch HT, Wang SM. Two PALB2 germline mutations found in both BRCA1+ and BRCAx familial breast cancer. *Breast Cancer Res Treat* 2015;151:219-224.

18. *I. Casey MJ, Bewtra C, Lynch HT, Snyder CL, Stacey M. Endometrial cancers in mutation carriers from hereditary breast ovarian cancer syndrome kindreds: report from the Creighton University Hereditary Cancer Registry with review of the implications. *Int J Gynecol Cancer* 2015;25:650-656.

19. *I. Cybulski C, Lubinski J, Huzarski T, Lynch HT, Randall SA, Neuhausen SL, Senter L, Friedman S, Ainsworth P, Singer C, Foulkes WD, Narod SA, Sun P, Kotsopoulos J.

Prospective evaluation of alcohol consumption and the risk of breast cancer in BRCA1 and BRCA2 mutation carriers. *Breast Cancer Res Treat* 2015;151:435-441.

20. *I. Metcalfe K, Lynch HT, Foulkes WD, Tung N, Kim-Sing C, Olopade OI, Eisen A, Rosen B, Snyder C, Gershman S, Sun P, Narod SA. Effect of oophorectomy on survival after breast cancer in BRCA1 and BRCA2 mutation carriers. *JAMA Oncol* 2015;1:306-313.

21. *I. Segev Y, Rosen B, Lubinski J, Gronwald J, Lynch HT, Moller P, Kim-Sing C, Ghadirian P, Karlan B, Eng C, Gilchrist D, Neuhausen SL, Eisen A, Friedman E, Euhus D, Ping S, Narod SA; Hereditary Breast Cancer Study Group. Risk factors for endometrial cancer among women with a BRCA1 or BRCA2 mutation: a case control study. *Fam Cancer*. In press.

22. Goodenberger ML, Thomas BC, Riegert-Johnson D, Boland CR, Plon SE, Clendenning M, Win AK, Senter L, Lipkin SM, Stadler ZK, Macrae FA, Lynch HT, Weitzel JN, de la Chapelle A, Syngal S, Lynch P, Parry S, Jenkins MA, Gallinger S, Holter S, Aronson M, Newcomb PA, Burnett T, Le Marchand L, Pichurin P, Hampel H, Terdiman JP, Lu KH, Thibodeau S, Lindor NM. PSM2 monoallelic mutation carriers: the known unknown. *Genet Med*. In press.

23. Tiwari AK, Roy HK, Lynch HT. Lynch syndrome in the 21st century: clinical perspectives. *QJM*. In press.

Molecular Mechanisms and Novel Targets in Cancer Program

Publications for Project 1 (Hansen):

1. Hammiller, B.O., El-Abaseri, T.B., Dlugosz, A.A., and Hansen, L.A. A method for the immortalization of newborn mouse skin keratinocytes. *Frontiers in Oncology,* 2015. doi: 10.3389/fonc.2015.00177

2. Shirley, S.H., Hammiller, B., Hansen, L.A., Crysup, B., Hudson, L.G., and Kusewitt, D.F. Role of the epidermal growth factor receptor in ultraviolet radiation induction of Snail family transcription factors. *J Dermatol Sci*, 76(2):149-51, 2014. doi: 10.1016/j.jdermsci.2014.09.001

Publications for Project 2 (Tu):

1. Zhou, T., Yu, M., Zhang, B., Wang, L., Wu, X., Zhou, H., Du, Y., Tu, Y., Chen, C., Wei, T. (2014) Inhibition of ancer cell migration by gold nanorods: molecular mechanisms and implications for cancer therapy. *Advanced Functional Materials* 24: 6922–6932 (IF: 11.805)
2. Zhou, T., Zhang, B., Wei, P., Du, Y., Zhou, H., Yu, M, Yan, L, Zhang, W, Nie, G., Chen, C., Tu, Y., Wei, T. (2014) Energy metabolism analysis reveals the mechanism of inhibition of breast cancer cell metastasis by PEG-modified graphene oxide nanosheets. *Biomaterials* 35: 9833-9843. PMID: 25212524 (IF: 8.557)
3. Barrio-Real, L., Benedetti, L.G., Engel, N., Tu, Y., Cho, S., Sukumar, S., Kazanietz, M.G. (2014) Subtype-specific overexpression of the Rac-GEF P-REX1 in breast cancer

is associated with promoter hypomethylation. *Breast Cancer Res1*6:441. PMCID: PMC4303123 (IF: 5.49)

4. Jiang, H., Xie, Y., Abel, P.W., Wolff, D.W., Toews, M.L., Panettieri. Jr R.A., Casale, T.B., Tu, Y. (2015) RGS2 repression exacerbates airway hyperresponsiveness and remodeling in asthma. *Am J Respir Cell Mol Biol* 53:42-49. PMID:25368964 (IF: 4.11)

Publications for Project 3 (Swanson):

A manuscript containing the data presented in Aim 4 was submitted to the *British Journal of Haematology* on July 8, 2015.

Publications for Project 4 (Chen):

1. Hu G, Gong AY, Wang Y, Ma S, Chen X, Chen J, Su C, Shibata A, Strauss-Soukup JK, Kristen M. Drescher KM, and Chen X-M. LincRNA-Cox2 promotes late inflammatory gene transcription through modulating SWI/SNF-mediated chromatin remodeling. (Under review by Cell Reports)

2. Tong Q, Gong AY, Lin C, Ma S, Chen J, Hu G, Strauss-Soukup JK, and Chen X-M. LincRNA-Cox2 Modulates TNFα-induced transcription of Il12b gene in epithelial cells through regulation of Mi-2/NuRD-mediated epigenetic histone modifications. (Under review by FASEB J)

Molecular and Cellular Mechanisms of Smoking-Related Lung Disease Program

Publications for Project 1 (Tu):

1. Zhou, T., Yu, M., Zhang, B., Wang, L., Wu, X., Zhou, H., Du, Y., Tu, Y., Chen, C., Wei, T. (2014) Inhibition of cancer cell migration by gold nanorods: molecular mechanisms and implications for cancer therapy. *Advanced Functional Materials* 24: 6922–6932 (IF: 11.805)

2. Zhou, T., Zhang, B., Wei, P., Du, Y., Zhou, H., Yu, M, Yan, L, Zhang, W, Nie, G., Chen, C., Tu, Y., Wei, T. (2014) Energy metabolism analysis reveals the mechanism of inhibition of breast cancer cell metastasis by PEG-modified graphene oxide nanosheets. *Biomaterials* 35: 9833-9843. PMID: 25212524 (IF: 8.557)

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Publications for Project 2 (Abel):

None

Publications for Project 3 (Oldenburg):

None

Development Program

Publications for Swanson project:

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Publications for Arouni project:

None

Publications for Hansen project:

1. Rao, V.H., Vogel, K., Yanagida, J.K., Marwaha, N., Kandel, A., Trempus, C., Repertinger, S.K., and Hansen, L.A. Erbb2 up-regulation of ADAM12 expression accelerates skin cancer progression. In press in *Molecular Carcinogenesis*, 2015

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Publications for Oldenburg project:

None