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TITLE: Hedgehog Signal Transduction Inhibitors in Breast Cancer Treatment and Prevention

PRINCIPAL INVESTIGATOR: Michael T. Lewis, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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13. ABSTRACT (Maximum 200 Words)

Mutations in at least two hedgehog signal transduction network genes leads to defects in mammary gland development. These mutations can cause enhanced hedgehog signaling in some organs. Data suggest activated hedgehog signaling may contribute to neoplasia and that hedgehog signalin inhibitors may be useful in breast cancer treatment. We find 1) that constitutive activation of hedgehog signaling by overexpression of the Smoothened effector protein in transgenic mice leads to increased proliferation and cancer-like developmental defects. 2) hedgehog signaling inhibitors such as cyclopamine slow or prevent breast cancer cell growth (MCF7 and MDA231) but do not alter "normal" cell (MCF10A). In addition, inhibitors show no measureable effect on normal mammary gland development. 3) Unexpectedly, Ptc1-induced defects are not inhibited or reverted by treatment with specific inhibitors of hedgehog signaling under the conditions tested. Our data suggest a role for hedgehog signaling in breast cancer and that hedgehog signaling inhibitors may be useful in breast cancer treatment.

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Table of Contents

Introduction

The hedgehog signal transduction network mediates cell-cell communication during normal embryonic development. Genetic mutation of hedgehog network genes can cause severe birth defects, basal cell carcinoma of the skin, and other tumors including medulloblastomas and glioblastomas of the brain.

Our recent work demonstrates a role for hedgehog signaling in mammary cancer and normal mammary gland development in the mouse. Loss-of-function mutations in two hedgehog network genes, *Patched-1 (Ptc-1)* and *Gli-2*, cause cancer-like lesions that closely resemble human ductal carcinoma *in situ* (DCIS. The lesions become invasive with age but, like basal cell carcinoma and medulloblastoma, are not stable upon transplantation.

The specific mechanism by which mutations in the hedgehog network lead to mammary lesions is not known. In basal cell carcinoma, loss of *Ptc-1* function or overproduction of either *Smoothened (Smo)* or one of the three hedgehog proteins, *Sonic hedgehog (Shh)*, leads to tumors. If the mechanics of the hedgehog signaling network are conserved between skin and mammary gland, a specialized skin derivative, these observations lead to the following hypothesis:

> *mutation of either the Ptc-1 or Gli2 genes results in improper activation of the signaling network via inappropriate activity of either Smo or the hedgehog proteins themselves. This improper signaling leads to loss of normal growth control and mammary lesion formation.*

We will use two approaches to test this hypothesis. First, we will construct a transgenic mouse line that expresses a constitutively activated form of *Smo* that signals independently of hedgehog protein binding and cannot be inhibited by *Ptc-1*. When expressed in the skin, this form of *Smo* promotes skin tumors. We expect that altered *Smo* gene will promote tumor formation and will therefore identify *Smo* as a mammary oncogene.

Second, we will use specific inhibitors of hedgehog protein signaling and determine whether these agents can reverse *Ptc-1-* , *Gli2* - or *Smo*-induced mammary lesions or lessen their severity. As controls we will examine the effects of these agents on well-characterized mouse tumor models. Agents will be delivered via surgical implantation of slow-release plastic pellets within the gland. Mammary glands will be examined for changes in structure and effects on lesion growth. Glands will also be assayed for changes in cell division (DNA synthesis) and cell death (apoptosis) as well as in expression of hedgehog network genes in response to treatment.

In addition, we will test the effect of these specific inhibitors on a panel of human breast cancer cell lines. It is possible that these inhibitors may affect the growth characteristics of a subset of human cancers thereby implicating the hedgehog network as a contributory factor in breast cancer onset or progression.

If hedgehog activity is responsible for, or participates in lesion formation or progression, we anticipate that treatment will reverse the formation of lesions or slow their growth. Such findings would justify expanded pre-clinical and clinical investigation of related hedgehog signaling inhibitors for potential therapeutic value in the treatment or prevention of human breast cancers.

Summary of results

Task 1. To determine whether constituitive activation of hedgehog signaling leads to mammary lesions in transgenic mice using an activated form of *Smo* that signals independently of hedgehog protein binding and is unresponsive to *Ptc-1* inhibition.

In collaboration with Dr. DeMayo, Director of the Baylor Mouse Genetics Core Facility, we have generated five MMTV-SmoM2 founder lines that all show consistent alteration of end bud morphology and histoarchitecture, as well as ductal dysplasias similar to those observed previously in the Ptc1/+ mice.

Immunofluorescence analysis demonstrates no changes in steroid hormone receptor expression but does show increased proliferation and co-expression of proliferation markers with steroid receptors, an early indication of neoplastic progression.

Task 2. To test the *in vivo* effect of specific hedgehog protein inhibitors on hedgehog network-induced lesions and the normal mammary gland.

Implants containing hedgehog signaling inhibitors had no demonstrable effect on normal mammary gland development. This finding is consistent with our current model for hedgehog regulation of mammary gland development in that we believe the network to be inactive during this phase of development normally.

Unexpectedly, we show no demonstrable effect of hedgehog signaling inhibitors on the severity of Ptc1-induced defects. This may be due to technical reasons (i.e. that the in vivo dose delivered by implantation is insufficient). However, recent data generated in our laboratory and others suggests a biological explanation. It appears that Ptc1 has at least four functions, some of which are hedgehog-related and some of which are hedgehog ligand independent. We are exploring the likelihood that Ptc1 defects are due to loss of a hedgehog independent function which would therefore be insensitive to cyclopamine inhibition.

Task 3. To test the *in vivo* effect of specific hedgehog protein inhibitors on hedgehog-independent lesions

We have entered into an expanded collaboration with Curis, Inc. and Genentech Inc. which recently developed small molecule inhibitors of Smo, on in vivo studies in transgenic mouse models (MMTV-c-ErbB2 and MMTV-Wnt1 models). We have not yet initiated these studies but still intend to perform them and will report on them to the DOD upon completion.

Task 4. To test the effect of hedgehog inhibitors on the growth and morphology of human breast cancer cell lines *in vitro*

We have completed dose-response growth curves for MCF7 (ER positive), MDA231, and MCF10A which show differential sensitivity to cyclopamine, KAAD-cyclopamine, and the Curis compounds.

We have completed colony forming assays for MCF7. Colony forming potential is decreased in a dose-dependent manner.

We have completed proliferation (MTT and BrdU incorporation) and apoptosis assays (TUNEL) for MCF7. Inhibitors affect proliferation rates but do not affect apoptosis rates.

Results are being prepared for publication. Based on these data, we are moving into in vivo preclinical models of breast cancer. A phase I clinical trial in patients with advanced breast cancer is in the design phase in collaboration with Dr. Jenny Chang of the Baylor Breast Center.

Key Research Accomplishments

With the exception of Task 3, the in vivo efficacy of compounds on mammary hyperplasias and tumors, the major goals of this project have been met. Results are entirely consistent with the initial hypothesis and strongly indicate that hedgehog signaling inhibitors should be considered for development in the treatment or prevention of breast cancer.

- Constitutive activation of hedgehog signaling in transgenic mice leads to mammary hyperplasia
- Cyclopamine and related compounds inhibit breast cancer cell proliferation and colony forming potential in a dose-dependent manner. Non-teratogenic steroidal alkaloids do not affect cell growth except at the high end of the dosages tested (50μM). Compounds affect proliferation but do not affect apoptosis.

Reportable Outcomes

Publications and Manuscripts

1. Lewis, M.T., and Veltmatt, J.M. (2004) Next stop, the twilight zone: hedgehog network regulation of mammary gland development. J. Mammary Gland Biol. Neoplasia 9:165-181.
2. Salomon, D.S. and Lewis, M.T. (2004) Embryogenesis and Oncogenesis: Dr. Jekyll and Mr. Hyde. J Mammary Gland Biol Neoplasia. 9:105-7.
3. Lewis, M.T. (2001) Hedgehog signaling in mammary gland development. J. Mammary Gland Biol. Neoplasia 6:53-66
4. Lewis, M.T., Ross, S., Strickland, P.A. Sugnet, C.W. Jimenez, E, Hui, C.C. and Daniel, C.W. (2001) The Gli2 transcription factor is required for normal mouse mammary gland development. Dev. Biol. .238:133-144

5. The *Hoxd10* Homeobox Gene Regulates Lactogenesis in the Mouse Mammary Gland. Michael T. Lewis[1*], Yael Friedman[2], Phyllis Strickland[3], Ellen M. Carpenter[4], and Charles W. Daniel[3] (submitted)
6. McManaman, J.L., Palmer, C.A., Zabaronik, W., Rizzoli, S., Fischer, A., Hanson, L., Lewis, M.T. and Neville, M.C. Regulation of lipid storage and secretion in the mouse mammary gland during secretory differentiation. (submitted)
7. Michael T. Lewis[1,2*], James McManaman[2], Linda Hanson[2], Valerie Sawicki[2], Gary B. Silberstein[3], Susan G. Hilsenbeck[1], and Margaret C. Neville[2] Hedgehog Signaling is Required for Functional Differentiation of the Mouse Mammary Gland (in preparation)
8. Harrington, N. and Lewis, M.T. Differential sensitivity of breast cancer cells to cyclopamine, a potent inhibitor of hedgehog signaling. (in preparation)
9. Moraes, R.C., Harrington, N., and Lewis, M.T. Activation of hedgehog signaling leads to mammary hyperplasia in transgenic mice. (in preparation)

Presentations

1. Baylor College of Medicine (Various)
2. Think Tank 12 (March 2002)
3. MD Anderson (Nov 2002)
4. Think Tank 13 (March 2003)
5. Gordon Research Conferences – Mammary Gland Biology (2000-2004)

Employment received and research opportunities.

Employment

7/01-present **Assistant Professor** – Baylor College of Medicine Breast Center and the Department of Molecular and Cellular Biology, Houston, TX 77030.

Research funding

ACTIVE

DAMD17-03-1-0571 (Lewis) 7/15/03-7/14/04
DOD
Combining Cell and Gene Therapy For Treatment Of Early Stage Breast Cancer

17580 (Lewis) 7/15/03-7/14/0
Susan Love MD Breast Cancer Research Foundation
Development of an Intraductal Cell and Gene Therapy Approach for Treatment of Early Stage Breast Cancer

P01 CA30195 (Osborne/Lewis) 12/1/03-11/30/08
NIH
Novel Gene Networks in Breast Development and Cancer, Project 4: The Ptc1 Hedgehog Receptor in Mammary Ductal Development and Progression to Neoplasia

PENDING

R01 CA30195 (Lewis) 6/1/04-7/31/09
NIH
The Ptc1 Hedgehog Receptor in Mammary Ductal Development and Progression to Neoplasia

No number 6/1/04-5/31/07
Susan G. Komen Foundation
In vivo analysis of the *Gli2* transcription factor gene, a candidate tumor suppressor in mammary stroma.

Conclusions

- Ductal development is not dependent on hedgehog signaling, and signaling must be inhibited actively for normal development to occur.

- Cyclopamine and related compounds are effective in inhibiting breast cancer cell growth in vitro. Compounds affect proliferation but not apoptosis.

- Ectopic hedgehog signaling leads to ductal dysplasia

Appendices

1. Curriculum vitae for Michael T. Lewis

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format on preceding page for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Michael T. Lewis	**Assistant Professor**

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

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
College of William and Mary, Williamsburg, Virginia USA	B.S.	1982-1986	Biology
University of California, Santa Cruz, California USA	Ph.D.	1989-1995	Biology
University of California, Santa Cruz, California USA	Post-doc.	1995-1998	Biology
University of Colorado, Denver, Colorado USA	Post-doc.	1999	Physiology and Biophysics

A. Positions

7/01-present **Assistant Professor** – Baylor College of Medicine Breast Center and the Department of Molecular and Cellular Biology, Houston, TX 77030.
Molecular genetics of mammary gland development and breast cancer in mouse and human. Focus on the function of the *hedgehog* signal transduction network and homeobox genes.

6/99-6/01 **Instructor** – University of Colorado School of Medicine, Denver, CO 80262. Department of Physiology and Biophysics.

1/99-6/99 **Postdoctoral Research Associate** – University of Colorado School of Medicine, Denver, CO 80262.
Department of Physiology and Biophysics. Laboratory of Dr. Peggy Neville.

7/95-12/98 **Post Graduate Researcher** – University of California, Santa Cruz, CA 95064. Laboratory of Dr. Charles Daniel.

9/89-6/95 **Teaching Assistant** – University of California. Department of Biology. Santa Cruz, CA 95064.
In: Genetics (5 quarters), Howard Hughes Summer Institute for Molecular Biology (5 quarters), Genetics Laboratory, Cell Biology, Neurobiology, and Virology.

9/89-6/95 **Graduate Researcher** – University of California. Department of Biology. Santa Cruz, CA 95064. Laboratory of Dr. Jerry Feldman.

7/88-8/89 **Research Scientist** – National Biomedical Research Foundation – Protein Information Resource (NBRF-PIR).

10/86-7/88 **Biologist** – National Biomedical Research Foundation – Protein Information Resource (NBRF-PIR).3900 Reservoir Rd., N.W., Washington, D.C., 20007.

B. Honors and Awards

1989 University of California Regents Fellowship

1992-Present	Charter member - Sigma Xi (Santa Cruz chapter)
2001	Organizer – Gordon Research Conference in Mammary Gland Biology Workshops
2001-2002	Member - University of California Breast Cancer Research Program - Pathogenesis Study Section
2001-Present	Member - San Antonio Breast Cancer Symposium Organization Committee
2003	Organizer – NIH Workshop on Homeobox genes in mammary gland development and tissue remodeling
2003-Present	Member - University of California Breast Cancer Research Program - Tumor Progression Study Section
2003-Present	Member – Dept. Defense Breast Cancer Research Program - Molecular Biology Genetics 2 Study Section

C. Publications

1. **Lewis, M.T.**, and Veltmart, J.M. (2004) Next stop, the twilight zone: hedgehog network regulation of mammary gland development. J. Mammary Gland Biol. Neoplasia 9:165-181.
2. Salomon, D.S. and **Lewis, M.T.** (2004) Embryogenesis and Oncogenesis: Dr. Jekyll and Mr. Hyde. J Mammary Gland Biol Neoplasia. 9:105-7.
3. **Lewis, M.T.** (2001) Hedgehog signaling in mammary gland development. J. Mammary Gland Biol. Neoplasia 6:53-66
4. **Lewis, M.T.**, Ross, S., Strickland, P.A., Sugnet, C., Jimenez, E., Hui, C-c. and Daniel, C.W. (2001) The *Gli2* transcription factor is required for normal mouse mammary gland development. Dev. Biol. 238:133-144
5. **Lewis, M.T.** (2000) Homeobox genes in mammary gland development and neoplasia. Breast Cancer Research 2: 158-169
6. Nguyen, D., Beeman, N., **Lewis, M.T.**, Schaack, J. and Neville, M.C. (2000) Intraductal injection into the mouse mammary gland. Methods in Mammary Gland Biology and Breast Cancer Research. M.M. Ip and B.B. Asch (eds.) Kluwer Academic/Plenum Publishers, New York. 259-270
7. **Lewis, M.T.**, Ross, S., Strickland, P.A., Sugnet, C., Jimenez, E. Scott, M.P. and Daniel, C.W. (1999) Defects in mouse mammary gland development caused by conditional haploinsufficiency of *Patched-1 (Ptc1)*. Development 126:5181-5193
8. **Lewis, M.T.**, Ross, S., Strickland, P.A., Snyder, C.J. and Daniel, C.W. (1999) Regulated expression patterns of *IRX-2*, an Iroquois-class homeobox gene, in the human breast. Cell Tissue Res. 296:549-554
9. **Lewis, M.T.** and Feldman, J.F. (1998) Genetic mapping of the *band (bd)* locus of *Neurospora crassa*. Fungal Genet. Newsl. 45:21.
10. **Lewis, M.T.**, Morgan, L.W., and Feldman, J.F. (1997) Analysis of *frequency (frq)* clock gene homologs: evidence for a helix-turn-helix transcription factor. Mol. Gen. Genet. 253:401-414
11. **Lewis, M.T.** and Feldman, J.F. (1996) Evolution of the *frequency (frq)* clock locus in fungi. Mol. Biol. Evol. 13:1233-1241
12. **Lewis, M.T.**, and Feldman, J.F. (1993) The putative *frequency (frq)* clock protein of *Neurospora crassa* contains sequence elements that suggest a nuclear transcriptional regulatory role. Protein Seq. Data Anal. 5: 315-323
13. Ron, D., Zannini, M., **Lewis, M.T.**, Wickner, R.B., Hunt, L.T., Graziani, G., Tronick, S.R., Aaronson, S.A., and Eva, A. (1991) A region of proto-*dbl* essential for its transforming activity shows sequence similarity to a yeast cell cycle gene, *CDC24*, and the human breakpoint cluster gene, *bcr*. The New Biologist 3: 372-379
14. **Lewis, M.T.**, Hunt, L.T., and Barker, W.C. (1989) Striking sequence similarity among sialic acid-binding lectin, pancreatic ribonucleases, and angiogenin: possible structural and functional relationships. Protein Seq. Data Anal. 2: 101-105

DATA COLLECTIONS:

Protein Sequence Database. Barker, W.C., Hunt, L.T., George, D.G., Yeh, L.S. Chen, H.R., Blomquist M.C., Seibel-Ross, E.I., Elzanowski, A., Bair, J.K., **Lewis, M.T.**, Marzec, C.R., Davalos, D.P. and Ledley, R.S. National Biomedical Research Foundation, Washington, D.C. (Updated quarterly) (1986-1989).

MANUSCRIPTS IN PREPARATION

Lewis, M.T., Friedman, Y., Strickland, P., Carpenter, E.M., Weiss, H., and Daniel, C.W. The *Hoxd10* homeobox gene regulate lactogenesis in the mouse mammary gland.

Lewis, M.T., Sawicki, V., Silberstein, G.B., Neville, M.C. and McManaman, J. Hedgehog signaling is required for lactation in the mouse mammary gland.

Harrington, N. and **Lewis, M.T.** Differential sensitivity of breast cancer cells to cyclopamine, a potent inhibitor of hedgehog signaling.

Moraes, R.C., Harrington, N., and **Lewis, M.T**. Activation of hedgehog signaling leads to mammary hyperplasia in transgenic mice.

D. Research Support.

Title: Novel Gene Networks in Breast Development and Cancer, Project 4: The Ptc1 Hedgehog Receptor in Mammary Ductal Development and Progression to Neoplasia
Agency: NIH P01 CA30195
Role: PI (Osborne/Lewis)
Period: 4/1/04-3/31/09
The major goals of this large Program Project are to identify and characterize the role of novel genetic pathways, which are found to be important in the normal breast, in the pathogenesis and progression of human breast cancer. The goal of Project 4 is to determine the role of Ptc1 in these processes.

Title: Novel Gene Networks in Breast Development and Cancer, Animal Handling Core
Agency: NIH P01 CA30195 (Osborne/Lewis)
Role: PI
Period: 4/1/04-3/31/09
The major goals of this large Program Project are to.identify and characterize the role of novel genetic pathways, which are found to be important in the normal breast, in the pathogenesis and progression of human breast cancer. The goal of Core C is to provide for purchase and housing of animals, and to provide specialized surgical and imaging support for all 5 projects included in this Program Project.

Title: Hedgehog Signal Transduction Inhibitors in Breast Cancer Treatment and Prevention
Agency: Department of Defense (IDEA) 17 00 1 0477
Role: PI
Period: 7/1/00-6/30/05
The major goals of this project are to determine whether constitutive activation of hedgehog signaling can lead to mammary lesions in transgenic mice using an activated form of *Smo* that signals independently of hedgehogs and is unresponsive to *Ptc-1* inhibition, to test the *in vivo* effect of specific hedgehog protein inhibitors on hedgehog network-induced lesions and the normal mammary gland, to test the *in vivo* effect of specific hedgehog protein inhibitors on hedgehog-independent lesions, and to test the effect of hedgehog inhibitors on the growth and morphology of human breast cancer cell lines *in vitro.*

Title: SPORE in Breast Cancer-Project 5: Genetic Expression Profile of Taxotere Versus AC Sensitivity
Agency: NIH (P50 CA58183)
Role: Co-Investigator
Period: 12/1/02-11/30/07
The main goal of this project is to identify, confirm and validate prospectively and retrospectively, two genetic pathways involved in the sensitivity and resistance of the two main treatment regimens in breast cancer, Taxotere (T) and Adriamycin plus cyclophosphamide (AC).

Title: Combining cell and gene therapy for treatment of early stage breast cancer
Agency: Department of Defense (DAMD17-03-1-0571)
Role: PI
Period: 7/1/03-6/30/04
The goal of this project is to define conditions under which genetically modified cells will persist when reintroduced to the mammary gland.

Title: Development of an Intraductal Cell and Gene Therapy Approach for Treatment of Early Stage Breast Cancer
Agency: Susan Love MD Breast Cancer Research Foundation
Role: PI
Period: 7/1/03-6/30-04
The goal of this pilot project is to perform a "proof of principle" experiment to determine whether a patient's own breast cells can be removed, genetically modified to perform a therapeutic function, and reintroduced intraductally to survive long-term to combat cancer.

Title: Induction of mammary cancer by signaling molecules
Agency: NCI (R01 CA85736 Anderson, PI)
Role: Co-Investigator
Period: 4/1/00- 3/31/05
The major goals of this project are to determine whether constitutive activation of either the prolactin receptor or one of its downstream effectors (Akt) will contribute to neoplastic progression or developmental defects in the mouse mammary gland.

Title: Functional Development of the Mammary Gland
Agency: NIH (PO1 HD38129 Neville, PI)
Role: Co-Project Leader/Animal Core Director
Period: 7/1/00-6/30/01 (6/30/05)

*Before relocating to Baylor College of Medicine, Dr. Lewis devoted 20% time as Co-Principal Investigator with Dr. Dean Edwards (UCHSC Department of Pathology) on a project to define the mechanisms of inhibition of milk secretion by progesterone during pregnancy and 20% time as the Animal Core Director for the program project group. He continues to collaborate with the group from the University of Colorado.