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PRINCIPAL INVESTIGATOR: Bogi Andersen, M.D.

CONTRACTING ORGANIZATION: University of California, Irvine
Irvine, California 92697-1875

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Bogi Andersen, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, Irvine
Irvine, California 92697-1875

E*Mail: bogi@uci.edu

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13. Abstract ***(Maximum 200 Words)*** ***(abstract should contain no proprietary or confidential information)***

We recently identified a novel transcription factor, LMO-4, which exhibits prominent expression in epithelial cells, including that of the breast. Since previous members of the LIM only (LMO) gene family are oncogenes in lymphocytes, we hypothesized that LMO-4 may play a role in mammary gland development and cancer. We have now shown that expression of LMO-4 is associated with undifferentiated cellular stage of breast epithelial cells, such as that found during lobuloalveolar development in pregnancy and in breast cancer. Yet, LMO-4 is not induced by estrogen, suggesting that it may participate in an estrogen-independent pathway. We have created transgenic mice in which we overexpress a dominant negative LMO-4 under the MMTV promoter (MMTV-engrailed-LMO4) to test the role of LMO-4 in mammary gland development. Our results show significant inhibition of lobuloalveolar development in these MMTV-engrailed-LMO4 transgenic mice, indicating that LMO-4 plays roles in proliferation and/or invasion of breast epithelial cells. Because these cellular features are associated with breast carcinogenesis and because LMO-4 is overexpressed in a subset of breast cancers, our studies implicate LMO4 as a possible oncogene in breast cancer.

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INTRODUCTION:

Understanding the mechanisms involved in regulating proliferation and differentiation of breast epithelial cells is important for further understanding the causes, diagnosis and treatment of breast cancer. We have recently identified a new protein called LMO-4, a member of a family of proteins that participate in gene regulation. Proteins belonging to this group have been shown to cause leukemia. We were therefore intrigued to find that LMO-4 is highly abundant in breast epithelial cells when these cells are proliferating. Our hypothesis is therefore that LMO-4 is involved in regulation of proliferation of breast epithelial cells in normal breast and in breast cancer. To test this hypothesis, we are pursing the following **Specific Aims**:

#1. Define the expression pattern of LMO-4 during normal mouse breast development and in human breast cancer.

#2. Define the role of LMO-4 in normal breast development and in breast cancer, using a mouse transgenic approach.

#3. Identify and characterize protein partners for LMO-4 in human breast tissue.

BODY:

Objective #1. **Define the expression pattern of LMO-4 during normal mouse breast development and in human breast cancer.**

a. Raise and purify LMO-4 antisera.

In previous progress report we had created LMO4 antisera from 3 guinea pigs and 2 rabbits and we had just started to characterize the antisera by immunohistochemistry. For testing, we had selected mouse embryos since these express LMO-4 at a high level in several locations. We described that in preliminary experiments we have had trouble obtaining consistent immunostaining on paraffin embedded tissue.

We have continued the characterization of the LMO4 antisera and despite trying a variety of different fixing conditions and fresh frozen tissues, we are not able to get consistent results. Other investigators in the field who have also tried to raise LMO4 antisera have experienced similar problems. In addition, recently available commercial LMO4 antisera, raised to peptide sequences both in the N- and C-termini of LMO4, have not proved useful for immunohistochemistry.

The LMO-4 antibodies are useful because they can detect recombinant LMO4 proteins and can be used for biochemical work. However, after this extensive, characterization and effort to use these antibodies for developmental studies, we have decided to resort to *in situ* hybridization with LMO4 cRNA probes and RNAse protection assays as described in our original proposal.

b. Obtain mouse embryos and pregnant mice at different developmental stages.

We have completed collecting fixed embedded mammary glands from mice at different developmental stages for use in immunohistochemistry and *in situ* hybridization studies. We have also completed isolation of RNA from mammary glands at different developmental stages for studying LMO-4 mRNA levels with RNAse protection assays.

c. Study LMO-4 expression during normal mammary gland development.

We have now used the samples obtained as described in **b.** to outline the expression of LMO-4 during mammary gland development and to correlate it with expression of the associated co-factor, Clim2. In these studies (Fig. 1), mRNA expression was analyzed by RNAse protection assays, using ^{32}P-labeled antisense riboprobes. Yeast tRNA was used as a negative control, and actin as internal control for variations in RNA

quantity and quality. The results show that LMO4 and Clim2 transcript levels are coordinately and greatly upregulated during mid-pregnancy, a stage in mammary gland development when epithelial cells are undergoing proliferation and invasion into the fat pad. Another expression peak is observed during lactation suggesting possible additional roles during this stage.

Our *in situ* hybridization studies also indicate that LMO-4 levels in mammary glands are highest during midpregnancy and become undetectable after weaning, and that LMO-4 is mainly expressed within the lobuloalveolar epithelial cells of the mammary gland (Fig. 3). Together, these experiments correlate the expression of LMO-4 and Clim2 with a stage in development when breast epithelial cells are relatively undifferentiated and undergoing proliferation and invasion. This expression pattern suggests the possibility that the LMO-4/Clim2 complex plays roles in maintaining proliferation and/or suppressing differentiation – two features that characterize breast cancer cells.

d. Obtain human breast and breast tumor samples.

We have obtained a panel of breast tumor sections through the UCI medical center. These can now be used to study expression of LMO4 in breast tumors.

e. Analyze expression of LMO-4 in normal human breast and breast cancer.

To evaluate LMO4 expression in breast cancer cell lines, we used RNAse protection assays with ^{32}P-labeled antisense riboprobes (Fig. 2). Yeast tRNA was used as negative control, and actin as an internal control. MCF-7 cells were grown in phenol red-free media, and stimulated with 20 nM 17-β estradiol or vehicle (control). The results show that expression of LMO-4 and Clim2 vary markedly between the three human breast cancer cell lines. LMO-4 is greatly overexpressed in one of the breast cancer cell lines, the MDA-MB-231 line, but low in MCF-7 cells. Transcript levels are not regulated by 17-β estradiol in MCF-7 cells. Because of difficulty with obtaining immunohistochemistry suitable LMO4 antisera, we have not been able to initiate studies of LMO4 expression in breast tumors. These studies, using *in situ* hybridization approaches, are now planned.

Ojective #2. **Define the role of LMO-4 in normal breast development and in breast cancer, using a transgenic approach.**

Two previously characterized members of the LMO-family, LMO-1 and LMO-2, have been found to be oncogenic. In humans these genes are overexpressed in lymphocytes due to fusion with the T-cell receptor in chromosomal translocations associated with acute lymphoblastic leukemia. These observations suggest that the LMO class of proteins plays roles in regulation of both proliferation and differentiation critical for organ development and that abnormalities in LMO-activity may lead to oncogenesis. Our hypothesis is that LMO-4 plays a role in normal breast development and that subversion of LMO-4 function or activity may contribute to formation of breast tumors.

We have elected to test our hypothesis using a transgenic approach, which allows us to test the role of LMO-4 in the context of the whole animal. We have made significant progress towards this goal. We have decided to create four sets of transgenic mice: one in which LMO-4 is overexpressed, one in which LMO-4 is converted into a "superactivator", one in which LMO-4 activity is inhibited, and a fourth one in which we have overexpressed a dominant negative form of the LMO-4-associated protein, CLIM.

a/b. Creation and testing of transgenic plasmids and microinjection of oocytes for establishing transgenic lines.

Overexpression of LMO-4: The LMO-4 cDNA fused in frame with a MYC epitope is expressed under the control of the mouse mammary tumor virus (MMTV) enhancer/promoter. This plasmid has been injected into oocytes and we have several positive founder mice.

Overexpression of LMO-4/VP-16 activation domain fusion: The HA-tagged LMO-4 cDNA was fused in frame with the activation domain of VP-16 and placed under the control of the MMTV promoter. We have established transgenic lines from founder mice described in last year's progress report.

Overexpression of LMO-4/engrailed repression domain fusion: The HA-tagged LMO-4 cDNA was fused in frame with the repression domain of engrailed and placed under the control of the MMTV promoter. The engrailed repression domain is dominant and overcomes transcriptional activation. Thus, we will create an artificial repressor that maintains the protein-protein interaction specificity of LMO-4. LMOs are thought to mediate their action by associating with DNA-binding proteins and simultaneously serving as docking proteins for the co-activator proteins CLIMs/Nli/Ldb that are thought to participate in transcriptional activation. The goal of our experiment is to overexpress the LMO-4/engrailed fusion to introduce a strong repressor domain into these DNA-protein complexes that will interfere with transactivation and instead repress LMO-4 target genes. We have established transgenic lines from founder mice described in last year's progress report.

Overexpression of a dominant negative CLIM molecule: The MYC tagged C-terminus of CLIM, which interacts with LIM domains, was placed under the control of the MMTV promoter. This approach has been previously used to interfere with the action of LIM factors. We have established transgenic lines from founder mice described in last year's progress report.

For the MMTV-LMO-4 mice, we are breeding the positive founders in preparation for selecting 2 to 3 lines that express LMO-4 in mammary epithelial cells, using immunohistochemistry with antibody against the MYC tag. For the other three transgenic lines, we are farther along and have already established 3 expressing MMTV-Engrailed-LMO4 lines, 3 expressing MMTV-VP16-LMO4 lines and 2 expressing MMV-dominant negative-Clim lines. Transgenic mice have been bred to C57BL/6 mice, which have low incidence of mammary cancer, to allow generation of sufficient female mice for analyses. So far, all transgenic mothers seem to be able to lactate.

In summary, we have made great strides towards conclusion of this most laborious and difficult part of our project.

c. Breeding and analyses of transgenic lines.

We have not yet obtained mice for analyses from the MMTV-LMO4 line, but we have been able to analyze mice from the other three transgenic lines. To study the effect of LMO-4 on mammary gland development, we have used whole mount analyses of mammary gland. These experiments show that the MMTV-Engrailed-LMO4 mice exhibit decreased lobuloalveolar development both during pubertal development and during early and mid-pregnancy (Fig. 4). These results are consistent with our hypothesis derived from the expression analyses and indicate that LMO4 is likely to play roles to promote invasion and/or proliferation of mammary gland epithelial cells. We have not observed a clear phenotype in the MMTV-VP16-LMO4 and MMTV-dominant negative-Clim lines.

Objective #3. **Identify and characterize protein partners for LMO-4 in human breast tissue.**

While LMO and LIM homeobox proteins are similar in that they are both localized to the nucleus, there is no evidence to suggest that the biological activity of LMOs is through direct DNA-binding. Insight into the biochemical mechanisms of actions for LMO proteins came from studies of LMO-1 and -2 in the hematopoietic system where it was found that LMO-2 interacts strongly with the bHLH domain of TAL1 and that these proteins, as well as Clim-2, exist in a complex in erythroid cells. These experiments suggest a model in which LMO factors can be tethered to DNA by

associating with DNA binding proteins, thus allowing the co-regulator CLIM to interact with transactivators that do not contain a covalently linked LIM domain.

We therefore propose that a LMO-4 and Clim-2 containing complex regulates gene activity in breast epithelial cells by associating with unidentified DNA-binding protein(s). The goal of the proposed experiments is to identify such factor(s). Specifically, we are interested in determining whether LMO-4 may interact with transcription factors or nuclear oncoproteins that have been shown to regulate differentiation and proliferation in normal and neoplastic breast.

a/b. Construction of yeast two hybrid libraries and screening with LMO-4 bait.

Library screening has been accomplished as described in last year's progress report. In a screen of a human breast library we isolated CLIM-2, human DEAF-1, the DNA-binding factor, Zn43 (1) and the splicosome, protein M4 and SPF27 (2). So far LMO factors have not been implicated in regulation of splicing, but recent data suggest that transcription factors may play a role in regulation of splicing. In addition, it is highly interesting that the gene expressing one of these factors, SPF27, was found to be highly amplified in human breast carcinoma cell lines. This gene has also been referred to as DAM1 (DNA amplified in mammary carcinoma) because it was isolated in screens designed to identify transcripts upregulated in human carcinoma cell lines (3).

c. Characterization of potential positive interacting factors.

We have initiated characterization of LMO-4 interacting factors by stably transfecting both LMO-4 and Clim-2 into MCF-7 breast cancer cell lines. The LMO-4 protein is tagged with Myc and the Clim-2 protein is tagged with HA, thus allowing specific immunoprecipitation of these proteins from breast cancer cell lines. For these studies, we have used Tetracyclin inducible vectors. This work has progressed well and we have already isolated several MCF-7 cell lines in which we can induce expression of LMO4 and Clim2 (Fig. 5). We can now proceed with immunoprecipitation studies to test whether the gene products isolated as described in a/b above interact with LMO-4 in breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS DURING LAST YEAR:

1. Definition of LMO4 and Clim2 gene expression during mammary gland development.
2. Outlining LMO4 and Clim2 expression in breast cancer cells and determining whether LMO4 and Clim2 are induced by estrogen.
3. Obtaining founder transgenic mice positive for MMTV-LMO4.
4. Establishment of transgenic lines and beginning analyses for MMTV-Engrailed-LMO4, MMTV-VP16-LMO4 and MMTV-dominant negative-Clim.
5. Showing that MMTV-Engrailed-LMO4 mice exhibit defective lobuloalveolar development, consistent with an important role for LMO4 in promoting proliferation and/or invasion of breast epithelial cells.
6. Creation of stable breast cancer cell lines expressing tagged LMO4 and Clim2 proteins, suitable for testing protein-protein interactions with immunoprecipitations.

REPORTABLE OUTCOMES TO DATE:

1. Development of antisera
2. Transgenic mouse models for LMO expression
3. Permanent breast cancer cell lines expressing tagged LMO4 and Clim2
4. A fellowship award (BC000553) was funded based on work on this project. This grant from the Army Med Rsearch & Dvelopment Command, entitled "Functional

Analysis of LIM Domain Proteins and Co-Factors in Breast Cancer", supports Dr. Ning Wang. Total amount is $150,000 over three years.

5. Manuscript in preparation: Wang, N., Kudryavtseva, E., Chen, I., Sugihara, T.M., McCormick, J., and **Andersen, B.** 2002. LMO-4 plays a role in lobuloalveolar development in the mammary gland. *In preparation.*
6. Abstract: Wang, N., Kudryavstseva, E., Chen, I., Sugihara, T., & **Andersen, B.** 2002. The potential role of a new LIM factor, LMO4, in breast cancer. Proceedings Era of Hope Meeting, Orlando Florida, September (Abstract P4-1).

CONCLUSIONS:

In summary, we have made significant progress on all three specific aims. Our results show that LMO-4 expression is associated with undifferentiated breast epithelial cells such as those found during mid-pregnancy and in breast cancer. The major achievement during the last year is the finding that interfering with LMO4 in breast epithelial cells leads to inhibition of lobuloalveolar development in mice. This finding, which we reported during the last Era of Hope meeting, strengthens our hypothesis that overexpression of LMO4 may contribute to breast carcinogenesis. With our work, we hope to generate new ideas about treatment of breast cancer, thus impacting on reducing the human/economic cost of breast cancer

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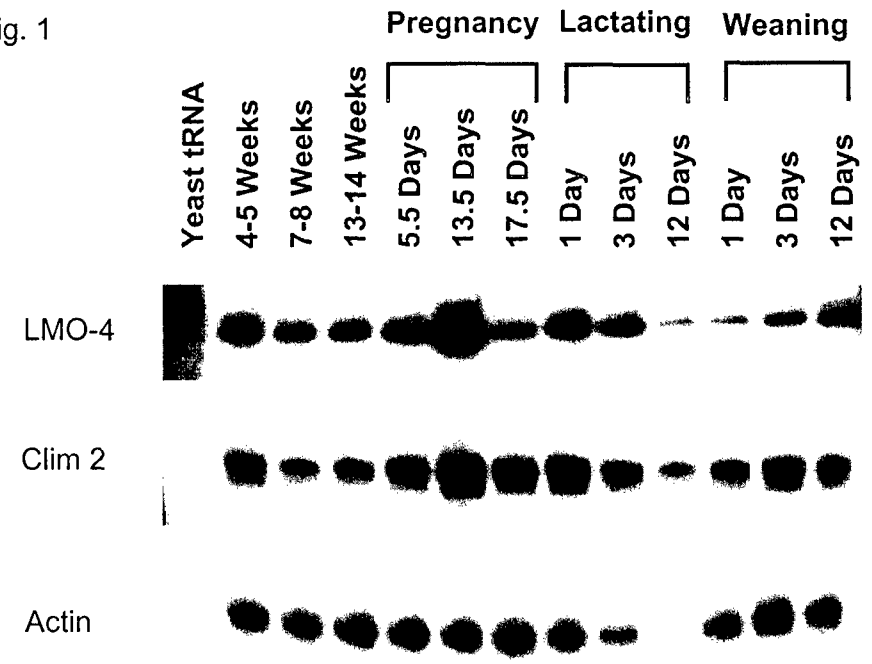
3. Nagasaki K., Maass N., Manabe T., Hanzawa H., Tsukada T., Kikuchi K., Yamaguchi K. Identification of a novel gene, DAM1, amplified at chromosome 1p13.3-21 region in human breast cancer cell lines. Cancer Lett 1999 Jun 1;140(1-2):219-26.

Fig. 1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Lactating
Weaning
Yeast tRNA
4-5 Weeks
7-8 Weeks
13-14 Weeks
5.5 Days
13.5 Days
17.5 Days
1 Day
3 Days
12 Days
1 Day
3 Days
12 Days
LMO-4
Clim 2
Actin

Fig. 2

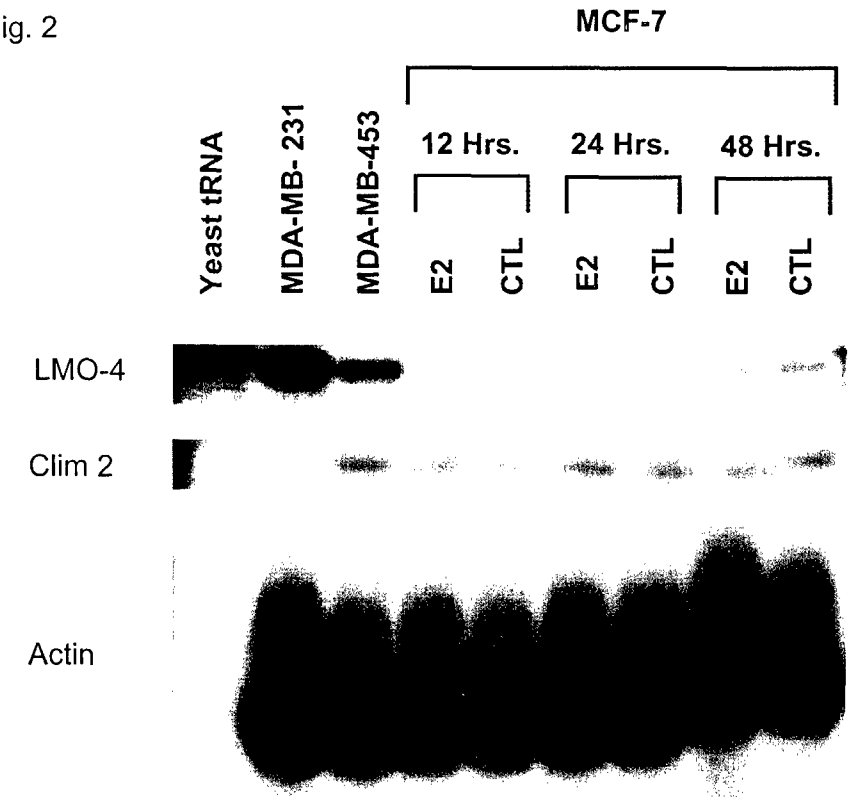

MCF-7
Yeast tRNA
MDA-MB- 231
MDA-MB-453
12 Hrs.
24 Hrs.
48 Hrs.
E2
CTL
E2
CTL
E2
CTL
LMO-4
Clim 2
Actin

Figure 3.

LMO-4 mRNA is highly expressed in lobuloalveolar epithelial cells of a 14.5 day pregnant mouse, with levels decreasing at day 18.5, just prior to delivery. LMO-4 is not detected by in situ hybridization at post-weaning day 7. Very little expression is observed in the stroma of the mammary gland.

LMO-4 IS EXPRESSED IN EPITHELIAL CELLS OF THE BREAST

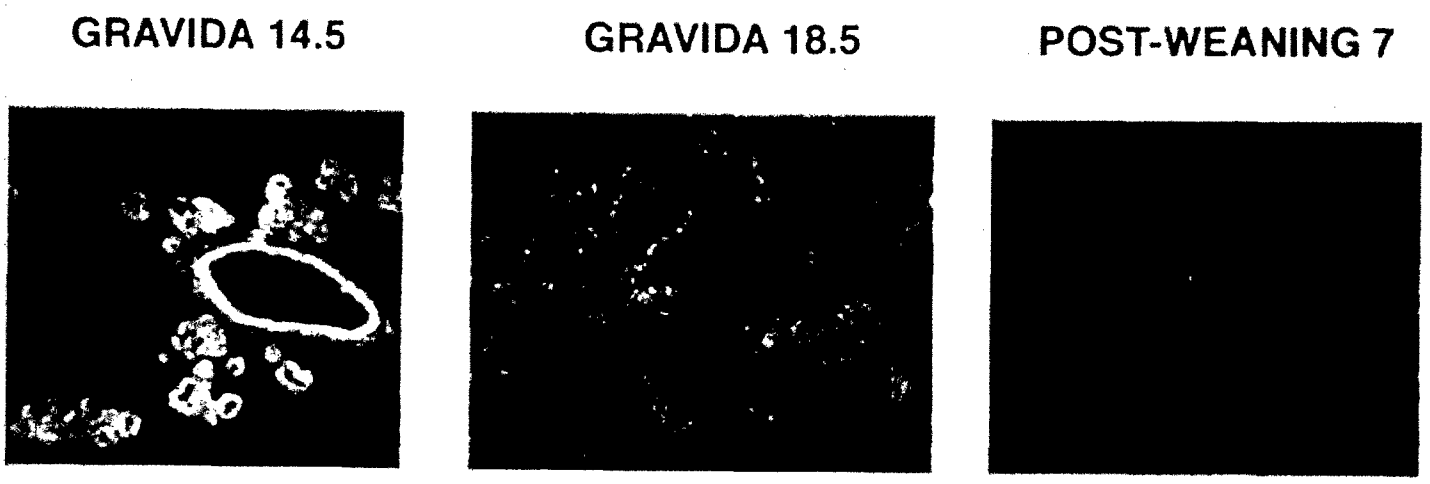
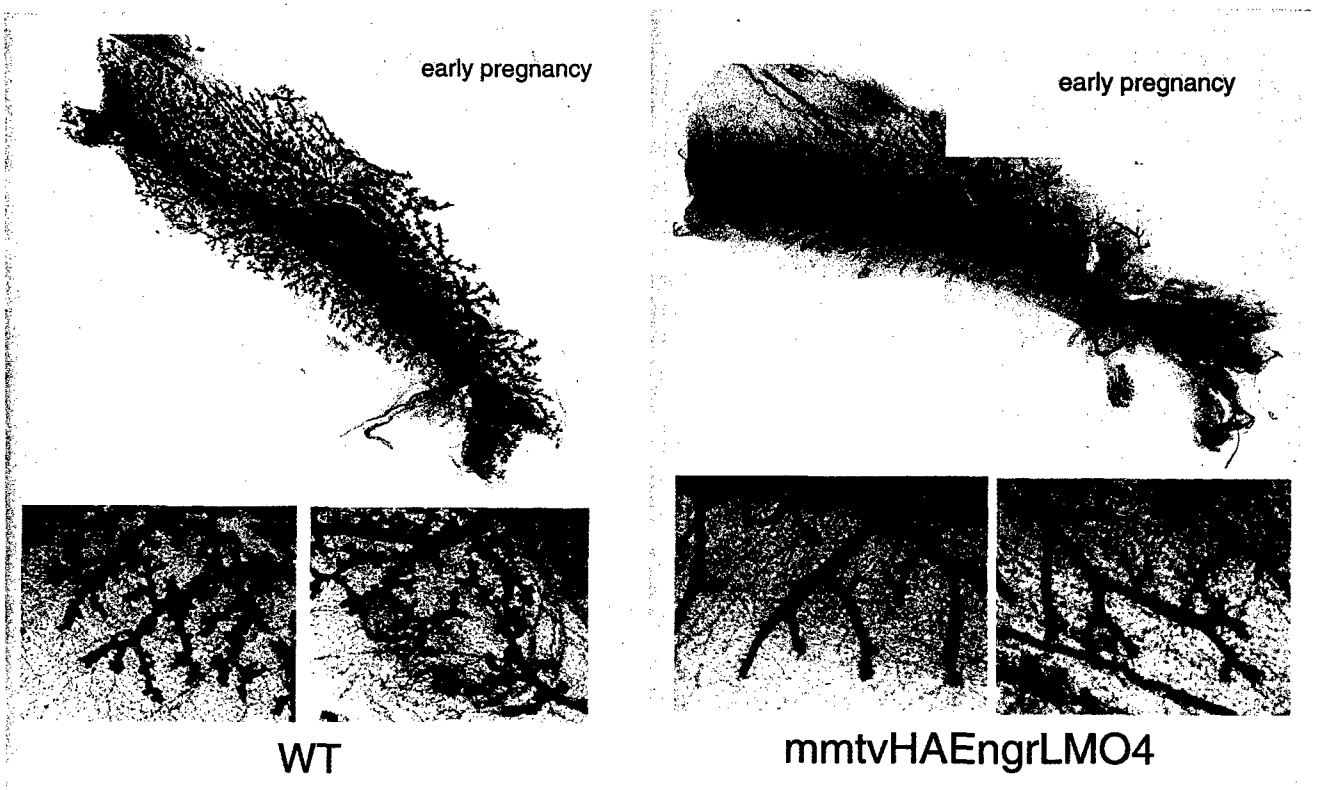


Figure 4.

The figure shows whole mount analyses of mammary glands at day 5.5 of pregnancy, comparing wild-type mice to MMTV-engrailed-LMO4 mice. Clear inhibition of lobuloalveolar development is observed in the transgenic mice. Similar findings were observed during mid-pregnancy (data not shown).



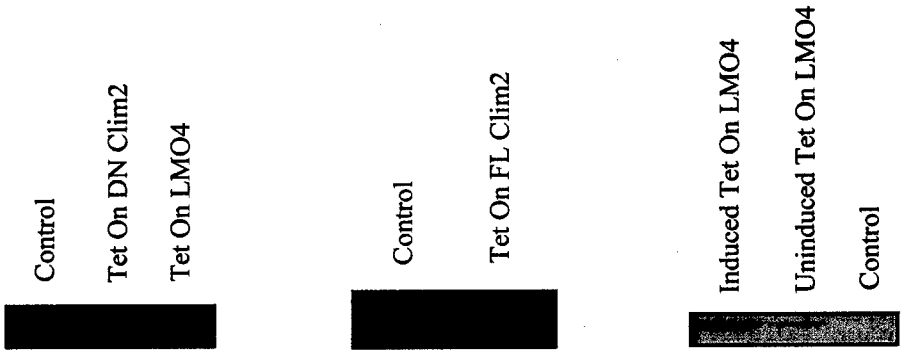


Figure 5. The left panel shows western blot detecting expression of DN-Clim and LMO-4 in pools of MCF-7 cells in response to tetracyclin. The middle panel shows tet-inducible expression of full length Clim2 under same condtions. The panel to the right shows results from a isolated MCF-7 cell clone where LMO-4 expression is induced by tet treatment for 48 hours.

THE POTENTIAL ROLE OF A NEW LIM FACTOR, LMO4, IN BREAST CANCER

Ning Wang, Elena Kudryavstseva, Irine Chen, Tod Sugihara, and Bogi Andersen

Department of Medicine, Division of Endocrinology, University of California, Irvine

bogi@uci.edu

Many properties of breast cancer cells, including increased proliferation and invasion, are common to epithelial cells of the developing mammary gland, suggesting that understanding of developmental control in normal mammary glands may provide important insights into the biology of breast cancer. This notion is supported by work in many organ systems, demonstrating that subversion of developmental control genes plays roles in carcinogenesis. LIM domain factors and associated co-regulators are important developmental regulators involved in pattern formation and organogenesis in a wide spectrum of organisms, including mammals. We isolated a LIM only factor, LMO-4, which is highly expressed in epithelial cells, including mammary epithelium. Interestingly, LMO factors are known to be oncogenic in lymphocytes where their overexpression causes acute lymphocytic leukemia.

We have studied expression of LMO-4 in mammary glands of mice and found that it is most highly expressed in proliferating mammary epithelial cells during pregnancy, suggesting that the LMO-4 gene may play a role in proliferation. Since LMOs do not bind to DNA it is likely that they regulate transcription by interacting with DNA-binding proteins and transcriptional co-regulators. To search for such factors, we have screened a human breast cDNA library with LMO-4 as bait in the yeast two hybrid system and found several potential interacting partners, including DNA-binding proteins, Clim/Nli/Ldb co-regulators and a splicing factor previously shown to be amplified in breast cancer cell lines. To test the role of LMO-4 in mammary gland biology, we have generated three lines of transgenic mice expressing under control of the MMTV promoter a) wild-type LMO-4, b) LMO-4 fused to the VP-16 transactivation domain and c) LMO-4 fused to the engrailed repression domain. Whole mount mammary gland analyses of these transgenic mice is in progress and preliminary results will be presented. Analyses of the EST databases indicate that LMO-4 is highly expressed in mammary carcinomas and we are in the process of evaluating its expression in breast cancer.

We conclude that LMO-4 may be an important regulator of mammary epithelial cells and propose a hypothesis that its high level expression in mammary tumors may play a role in mammary carcinogenesis.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0182 and DAMD17-01-1-0183 supported this work.