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13. ABSTRACT (Maximum 200 Words)

We 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 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 ductal development in virgin mice and alveolar development of pregnant 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. We have also created stable breast cancer cell lines in which we can induce expression of LMO4 and Clim2, and identified several cancer-related genes that are regulated by LMO4. In addition, we have shown that LMO4 interacts with the transcription factor GATA3 in breast cancer cells. We propose that LMO4 is involved in regulation of breast cancer cells by interacting with GATA3.

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Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....4

Key Research Accomplishments.....7

Reportable Outcomes.....7

Conclusions.....8

References.....8

Appendices.....10

INTRODUCTION

LIM only factors, LMOs, are nuclear proteins composed of two LIM domains and little other sequence. Two of the founding members of this family are act as oncogenes in lymphocytes. We have recently identified a new member of this family, LMO-4, which is highly expressed in proliferating epithelial cells, including those of the breast. In addition, LMO-4 has been shown to be highly expressed in ductal breast carcinoma [1]. Two mechanisms of action have been proposed for LMOs. First, they may bind the LIM homeodomain co-activator CLIM thereby interfering with the activity of LIM homeodomain proteins. Second, LMOs may associate with DNA-binding proteins, thus attracting the coactivator CLIM to DNA. Our hypothesis is that LMO-4 plays a role in regulation of breast epithelial cell proliferation and that subversion of its function may play a role in breast cancer. Further, that LMO-4 acts, at least in part, by associating with DNA-binding proteins.

Our specific aims are: #1. To test the potential role of the LMO-4/CLIM complex in breast development and breast cancer by using the MMTV promoter to overexpress LMO-4 and a dominant negative form of CLIM in breast epithelial cells. #2. We have recently identified a KRAB Zinc finger transcription factor, Znf43, as a potential interacting partner of LMO-4. We propose to test the relevance of this interaction using human breast carcinoma cell lines. We will test the effect on proliferation and tumor growth.

BODY

Task 1. **Test the effect of overexpressing LMO-4 and a dominant negative CLIM in the breast of transgenic mice.**

1. Create and characterize transgenic constructs.

The initial plan was to create two lines of transgenic mice: MMTV-LMO4 and MMTV-dominant negative-Clim. In addition, I have worked on a third transgenic line where we have created a dominant negative LMO4 molecule by linking LMO4 to the Engrailed repression domain. This construct is referred to as MMTV-Engrailed-LMO4. All three transgenic constructs have been created and characterized.

2. Injecting transgenic constructs into oocytes.

Accomplished during last reporting period, see previous Progress Report.

3. Analyses of founder mice.

Accomplished during last reporting period, see previous Progress Report.

4. Generating transgenic progeny.

Transgenic progeny were generated for the MMTV-dominant negative-Clim and MMTV-Engrailed-LMO4 mice prior to last Progress Report. Progeny were generated for the MMTV-LMO4 mice during the current period.

5. Analyses of transgenic mice.

To test the effect of the Engrailed-LMO4 molecule on mammary gland development, we placed it under control of the MMTV promoter (Fig. 1A), which directs high expression in epithelial cells of mammary glands in transgenic mice and has been extensively used for this purpose [2-5]. The fusion protein was HA tagged to allow its immunodetection in mammary glands. Of five transgenic lines, three independent lines expressed the transgene in mammary gland epithelial cells. Expression of the transgene was found both in virgin and pregnant mammary glands (Fig. 1B) and was predominantly nuclear (Fig. 1C).

To evaluate the effects of expressing the Engrailed-LMO4 fusion protein in mammary gland epithelial cells, we examined mammary gland development by whole mount analyses in transgenic mice and compared them to wild-type littermates. Development of transgenic mammary glands of virgin mice was normal at 3 to 4 weeks (data not shown), but at 6 weeks a mild delay in the progression of ductal development was evident (Fig. 2). At 8 weeks, most transgenic mammary glands were normal (Fig. 2, compare WT panel and lower TG panel at 8 weeks), although we did observe occasional abnormality at that stage (Fig. 2, compare WT panel and upper TG panel at 8 weeks). These data indicate that the Engrailed-LMO4 fusion protein causes a transient delay in mammary gland development of virgin mice. In pregnant transgenic mice, a clear delay in alveolar development was evident at day 5.5 (Fig. 3A and B, left panels); this delay, however, was later overcome and by day 15.5, lobuloalveolar development was essentially normal (Fig. 3B, middle panels). No abnormalities were observed during lactation (Fig. 3B, right panels) and transgenic females were able to nurse normal-size litters. In conclusion, expression of the dominant negative Engrailed-LMO4 fusion protein in the mammary glands of mice results in the slowing of ductal development in virgin mice and a transient inhibition of alveolar development during pregnancy. 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-dominant negative-Clim lines. Analyses of the MMTV-LMO4 lines showed that this transgene was expressed at very low level, precluding analyses of these mice. To troubleshoot this, we have generated MCF-7 breast cancer cell lines in which we can induce expression of LMO4. These cell lines will allow us to investigate the effect of LMO4 overexpression in breast cancer.

To test the effect of LMO4 in mammary epithelial cells, we have profiled expression in MCF-7 cells expressing LMO4 in a conditional manner, using the cell lines described in described below (Fig. 6). For these experiments, we selected 3 independent cell clones and profiled expression under basal conditions (control conditions, in the presence of doxycycline) and under induced conditions (LMO4 expression, 7 days after doxycycline withdrawal). To decrease variability, RNA samples from two independent experiments were pooled for each of the three cell clones. We hybridized to U133A Affymetrix chips, which contain 15,000 known or annotated human genes, and analyzed the data with the Cyber-T program, which was developed at UCI [6]. This statistical data package, which is especially suitable for pairwise comparisons, uses a Bayesian statistical framework to determine the local confidence (p-values) based on the *t*-test distribution of individual gene measurements. Thus, for each experimental condition, we can obtain: a

mean expression level, a fold-change between control and experimental condition, and a *t*-value to establish the confidence level of the observed difference in expression of a particular gene between control and experimental conditions.

Using probability criteria of $p \leq 0.01$, 318 of 15,000 genes were altered after LMO4 induction. Of these 318 genes, 182 are upregulated and 136 are downregulated. Interestingly, this experiment suggests that LMO4 cannot only stimulate gene expression, but it also can repress a group of genes. Table 1 shows a list of the top genes (listed in order of increasing p value) showing differential expression after induction of LMO4 in MCF-7 cells. Several of the genes regulated by LMO4 have been implicated in oncogenesis of epithelial cells, including breast cancer cells. Among these genes are: (1) The midkine/cytokine **Pleiotrophin (PTN),** which was purified from conditioned media of the highly malignant (also a high LMO4 expresser) MDA-MB-231 breast cancer cell line [7]. PTN is expressed in breast cancer and has been shown to act as an important paracrine and angiogenic factor for human breast cancer [8, 9]. It has growth-promoting and transforming activity on fibroblasts and epithelial cells, and mitogenic activity on endothelial cells [10]. Furthermore, PTN induces tube formation on endothelial cells and angiogenesis *in vitro* and in the rabbit corneal assay [9]. (2) **Metallothioneins 1X, -1L and –1H**, which are overexpressed in portion of breast cancer cases [11] and predict a poor prognosis [12]. Metallothioneins are thought to protect against oxidative stress and apoptosis [13]. (3) **FGF receptor 4,** which is overexpressed in breast cancer cell lines [14]. Several FGFs are known to be oncogenes in mouse mammary cancer [15]. At least one FGF, capable of activating FGFR-4, is overexpressed in human breast cancer [16]. (4) **Interleukin 8.** Its expression correlated with increased bone metastasis in a population of human breast cancer cells [17], and was demonstrated to stimulate osteoclastogenesis and bone resorbtion [18]. Moreover, IL-8 increases the invasiveness of ER-positive breast cancer cells by two fold, thus confirming the invasion-promoting role of IL-8 [19]. Further evidence for the important role of IL-8 in breast cancer comes from a study showing that combined administration of antibodies to human IL-8 and epidermal growth factor receptor results in increased antimetastatic effects on human breast carcinoma xenografts [20]. Additional up-regulated genes of interest are **BMP-7**, the **Ret proto-oncogene** and **Wnt-2B**. Among the selected down-regulated genes is **Tenascin X.** While its role in human breast cancer is unclear, tenascin X knockout mice exhibit increased tumor invasion and metastasis [21], consistent with a model in which tenascin inhibits migration and invasion. Additional down-regulated genes of interest are **Pleimorphic adenoma gene 1** and **Interleukin 17**.

In summary, LMO4 alters the expression of several genes involved in oncogenesis. We will now validate these targets using independent methods such as quantitative PCR and test whether Clim2 regulates the same panel of genes.

Task 2. Test the relevance of LMO-4/Znf43 interactions in breast cancer cell lines.

1. Creating constructs and cell lines transfections.

We initiated characterization of LMO-4 interacting factors by creating LMO-4 and Clim-2 expressing vectors that can be induced by tetracycline (Tet-on system), as described in the last Progress Report. However, expression in these cell lines was not

stable and therefore, we have switched to the Tet-off system. 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. The construction of both vectors was completed and several cell lines were generated (Figs. 6 and 7).

2. Performing in vitro interaction assays in vitro and in cells.

The LMO2 oncogene, which is highly related to LMO4 is known to interact with GATA factors [22]. This suggested the possibility that LMO4 might also interact with GATA factors. Of GATA factors, GATA3 has been shown to be expressed in breast cancer cells [23]. We therefore evaluated its expression during mammary gland development in the mouse (Fig. 4) and demonstrated that GATA3 is expressed throughout mammary gland development, and especially highly during pregnancy. In addition, we tested whether LMO4 is capable of interacting with GATA3, using co-immunoprecipitations of extracts from the MCF-7 cells stably expressing Myc tagged LMO4. In this assay, we were able to demonstrate an *in vivo* interaction between GATA3 and LMO4 (Fig. 5). In summary, LMO4 may act by associating with GATA3 in normal and malignant mammary epithelial cells. In future experiments, we plan to characterize this interaction further.

3. Creating stable cell lines for analyses.

The vectors described above have been stably introduced into MCF-7 breast cancer cell lines. This work has progressed well and we have already isolated several MCF-7 cell lines in which we can induce expression of LMO4 (Fig. 6) and Clim2 (Fig. 7) (Tet-off system).

KEY RESEARCH ACCOMPLISHMENTS DURING LAST YEAR

1. Showing that MMTV-Engrailed-LMO4 mice exhibit defective ductular development in virgin mice and defective alveolar development in pregnant mice.
2. Identifying several target genes of LMO4 using microarray analyses, many of which are involved in oncogenesis.
3. Demonstrating expression of GATA3 in mammary glands of mice.
4. Demonstrating an in vivo interaction between LMO4 and GATA3.
5. Creation of stable breast cancer cell lines expressing tagged LMO4 and Clim2 proteins, using the Tet-off system.

REPORTABLE OUTCOMES TO DATE

1. Transgenic mouse models for LMO expression
2. Permanent breast cancer cell lines expressing tagged LMO4 and Clim2 in an inducible manner
3. Manuscript in press: **Wang, N.**, Kudryavtseva, E., Chen, I., Sugihara, T.M., McCormick, J., and Andersen, B. 2003. Expression of an Engrailed-LMO4 fusion protein in mammary epithelial cells inhibits mammary gland development in mice. *Oncogene,* in press.

4. 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).
5. Abstract: **Wang, N.**, & Andersen, B. 2003. The potential role of a new LIM factor, LMO4, in breast cancer. California Breast Cancer Research Program meeting in San Diego, California, September.

CONCLUSIONS

In summary, I have made significant progress on both specific aims, and my training in breast cancer biology has been greatly enhanced. I have already published two abstracts and a first-author paper describing some of my findings is in press in *Oncogene*. 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 achievements during the last year are (1) the finding that interfering with LMO4 in breast epithelial cells leads to inhibition of ductular and alveolar development in mice, and (2) the identification of several LMO4 target genes, many of which are involved in oncogenesis. In addition, I have identified GATA3 as an LMO4-interacting transcription factor. Our findings strengthen the 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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APPENDIX COVER SHEET

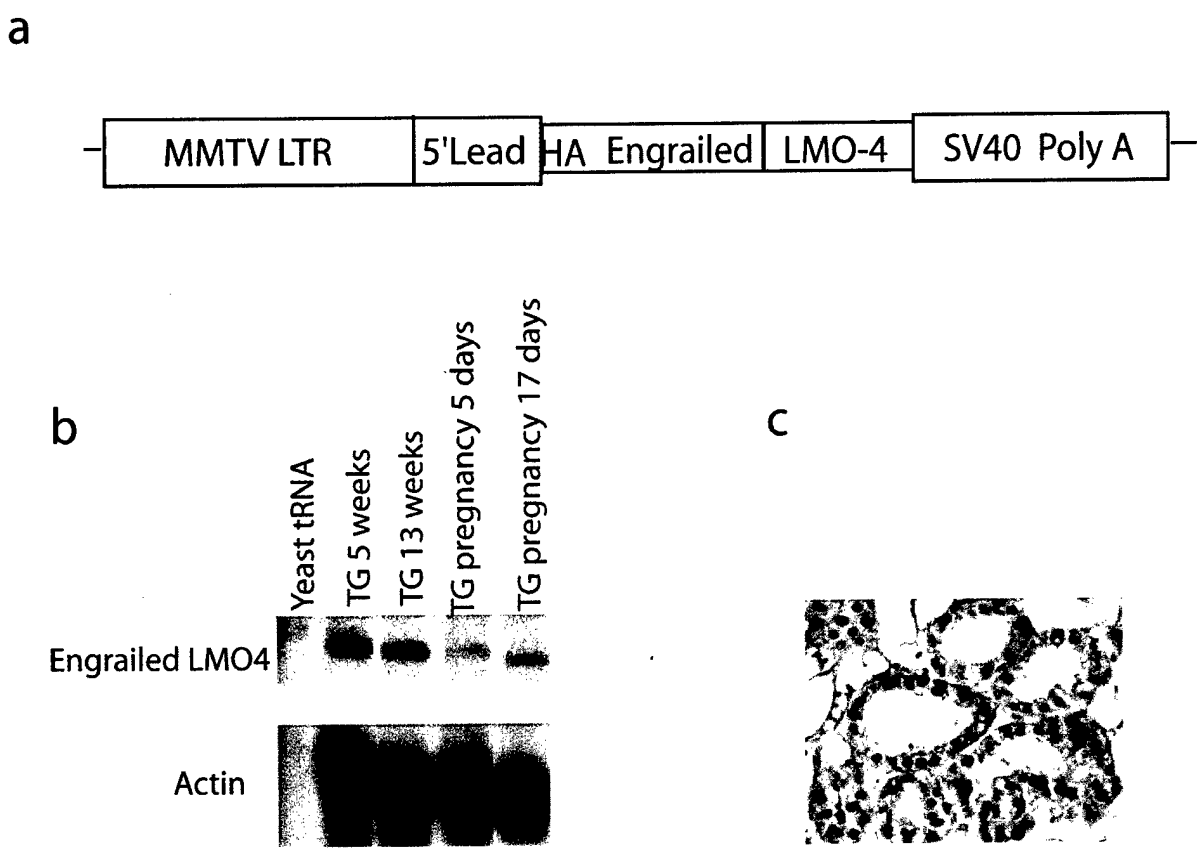

Figure 1. *The Engrailed-LMO4 transgene.* ***(a)*** *A schematic of the transgene.* ***(b)*** *RNAse protection assays showing expression of the Engrailed-LMO4 transgene at the indicated developmental time points.* ***(c)*** *HA immunostaining of mammary gland (day 1 of lactation) from MMTV-HA-Engrailed-LMO4 mice.*

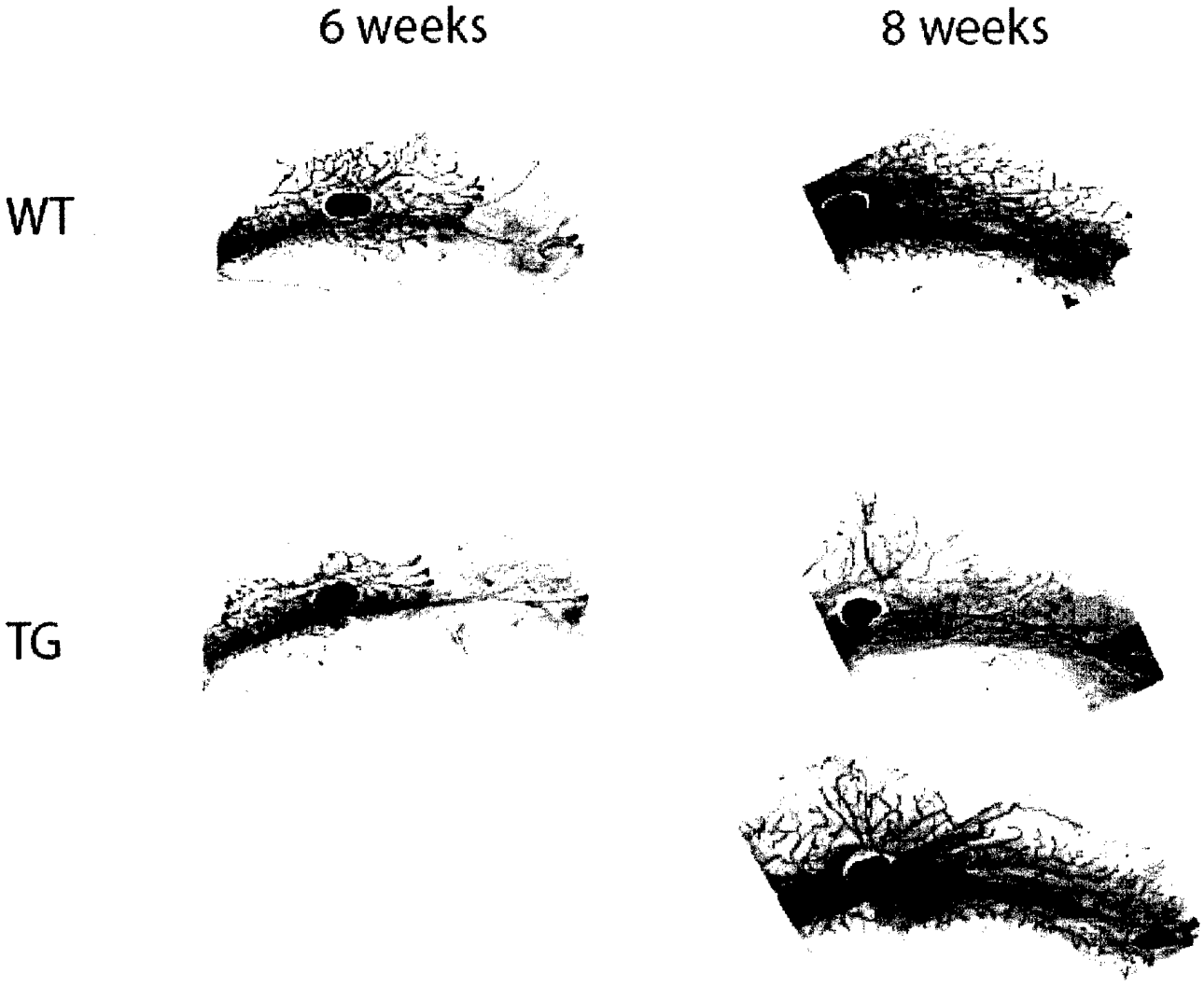

Figure 2. *The effect of the Engrailed-LMO4 fusion protein on mammary gland development in virgin mice. Whole mount staining of the fourth inguinal mammary glands from MMTV-HA-Engrailed-LMO4 (TG) mice and littermate wild-type (WT) controls at the indicated developmental stages.*

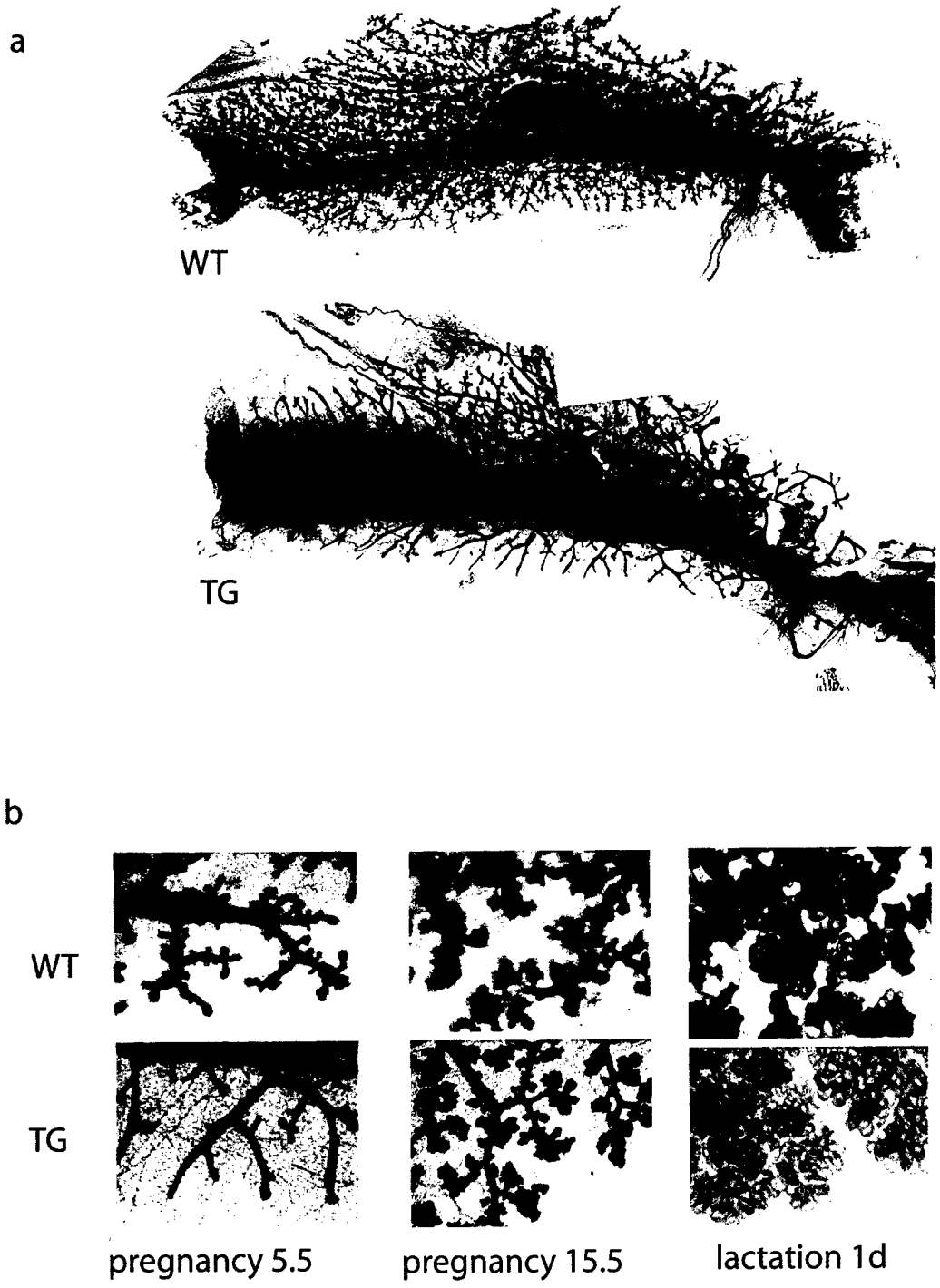

Figure 3. *The effect of the Engrailed-LMO4 fusion protein on mammary gland development during pregnancy.* ***(a)*** *Whole mount overview of mammary glands from 5.5 day pregnant mice comparing wild-type (WT) and transgenic (TG) mice.* ***(b)*** *Whole mount analyses in higher magnification from the indicated developmental stages.*

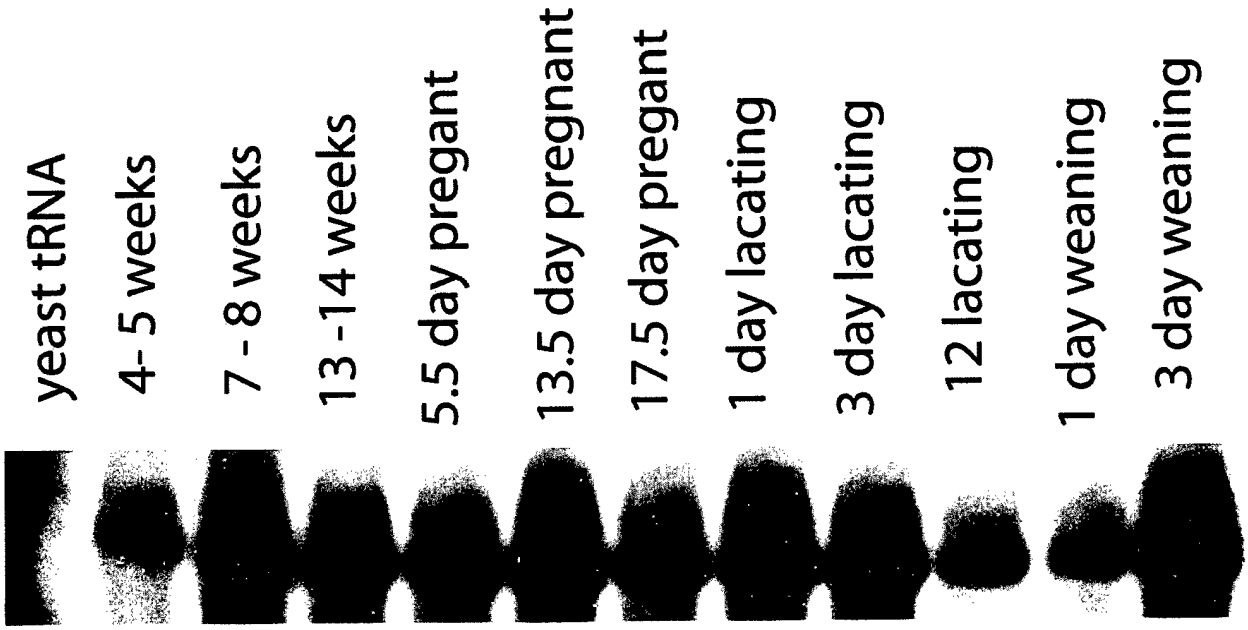

Figure 4. *Expression of GATA3 in mammary glands from the indicated stages, using RNAse protection assays.*

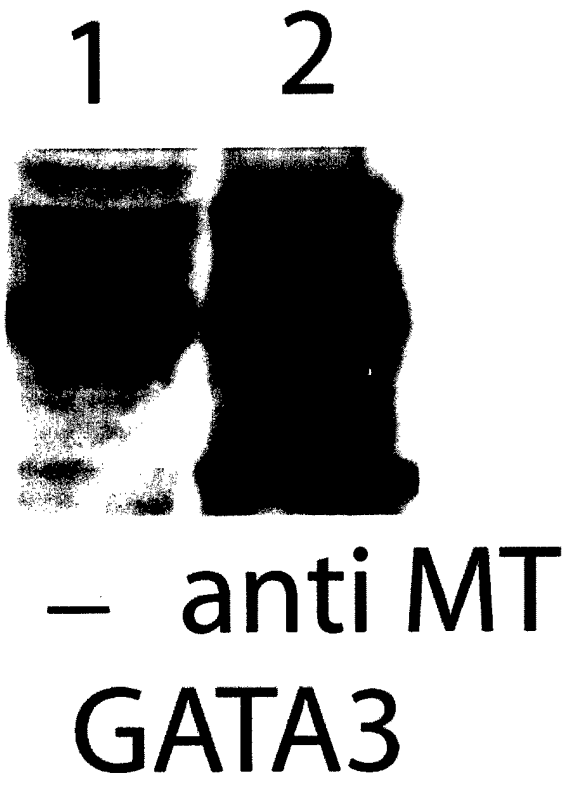

Figure 5. Co-immunoprecipitation showing interaction between LMO4 and GATA3 in MCF-7 breast cancer cells. Extracts from MCF-7 cells expressing Myc tagged LMO4 were analyzed by SDS gel electrophoreses without (lane 1) or after immunoprecipitation with a Myc antibody (lane 2). Western blot was probed with GATA3 antibody. Immunoprecipitation with Myc antibody precipitates GATA3, indicating interaction between LMO4 and GATA3.

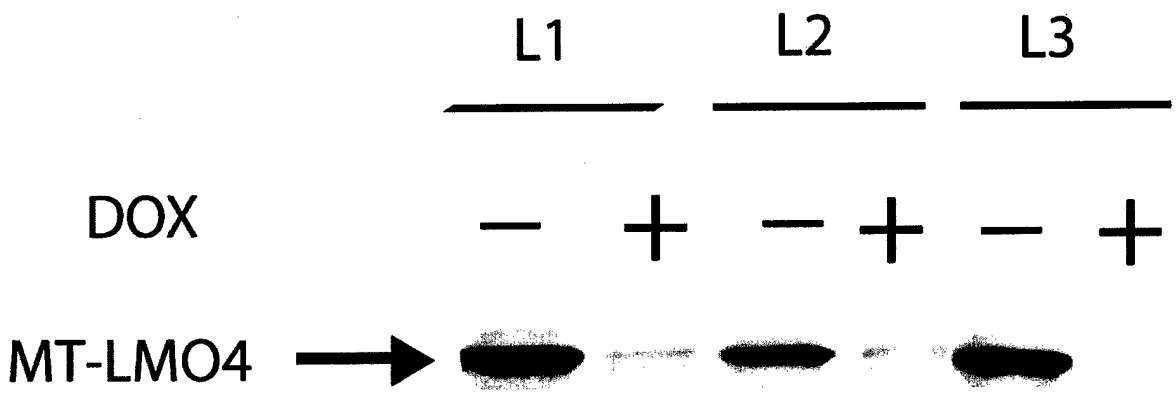

Figure 6. *Representative Tet-Off LMO4-inducible MCF-7 cell clones. Shown are three independent MCF-7 cell clones, with and without doxycycline (DOX) analyzed by a western blot, using Myc antibody.*

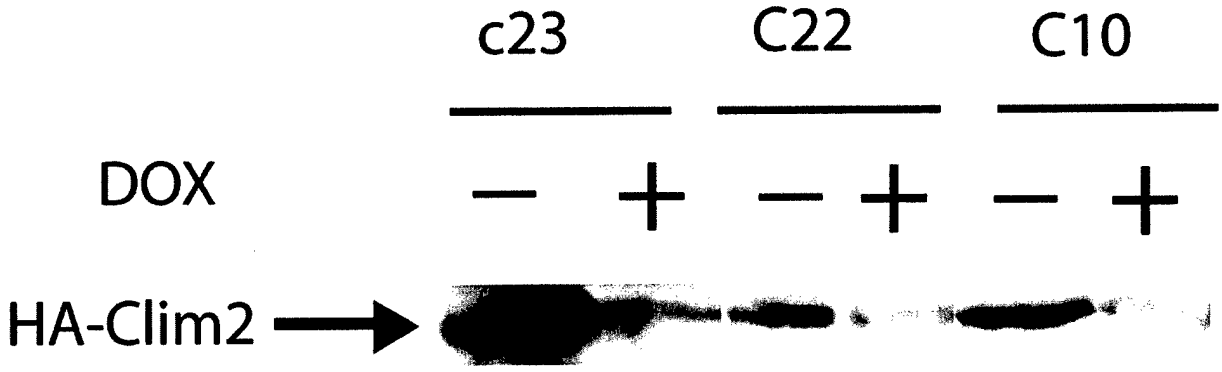

Figure 7. *Representative Tet-Off MCF-7 cell clones. Shown are three independent Clim2-inducible MCF-7 cell clones, with and without doxycycline (DOX) analyzed by a western blot, using HA antibody.*

Expression of an Engrailed-LMO4 fusion protein in mammary epithelial cells inhibits mammary gland development in mice

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Key words: LMO4, mammary gland development, heregulin, Engrailed fusion protein

Summary

LIM domain factors and associated co-factors are important developmental regulators in pattern formation and organogenesis. In addition, overexpression of two LIM-only factors (LMOs) causes acute lymphocytic leukemia. The more recently discovered LMO factor LMO4 is highly expressed in proliferating epithelial cells, and frequently overexpressed in breast carcinoma. Here we show that while LMO4 is expressed throughout mammary gland development, it is dramatically up-regulated in mammary epithelial cells during mid-pregnancy. The LMO co-activator Clim2/Ldb1/Nli showed a similar expression pattern, consistent with the idea that LMO4 and Clim2 act as a complex in mammary epithelial cells. In MCF-7 cells, LMO4 transcripts were up-regulated by heregulin, an activator of ErbB receptors that are known to be important in mammary gland development and breast cancer. To test the hypothesis that LMO4 plays roles in mammary gland development, we created an Engrailed-LMO4 fusion protein. This fusion protein maintains the ability to interact with Clim2, but acts as a dominant repressor of both basal and activated transcription when recruited to a DNA regulatory region. When the Engrailed-LMO4 fusion protein was expressed under control of the MMTV promoter in transgenic mice, both ductular development in virgin mice and alveolar development in pregnant mice were inhibited. These results suggest that LMO4 plays a role in promoting mammary gland development.

The LIM motif, a cysteine-rich zinc-coordinating domain that mediates protein-protein interactions, was originally discovered as a component of homeodomain transcription factors (reviewed in Bach, 2000). A second class of LIM domain transcription factors, composed almost entirely of two tandem LIM domains, is referred to as LIM-only (LMO) proteins. Two members, LMO1 and LMO2, are oncoproteins found at sites of chromosomal translocations in acute T-cell leukemia (Rabbitts et al., 1999). LMO proteins do not bind DNA directly, but regulate gene transcription by associating with other transcription factors. This model is supported by studies showing that LMOs, through their LIM domains, exist in a stable complex with helix loop helix (HLH) heterodimeric partner proteins that include TAL1(SCL)/E12 (Bao et al., 2000; Herblot et al., 2000; Larson et al., 1996; Mead et al., 2001; Ono et al., 1998; Osada et al., 1995; Osada et al., 1997; Valge-Archer et al., 1994; Visvader et al., 1997; Wadman et al., 1994; Wadman et al., 1997), and GATA factors (Mead et al., 2001; Ono et al., 1998; Osada et al., 1995; Wadman et al., 1997). In addition, LIM domains of LIM homeodomain and LMO proteins interact strongly with cofactors, including the coactivators Clim1 and Clim2/Lbd1/NLI (Agulnick et al., 1996; Bach et al., 1997; Bach et al., 1999; Jurata et al., 1996; Visvader et al., 1997), which confer transcriptional activation and promote synergism between DNA-binding proteins (Bach, 2000).

Based on the prominent expression of Clim2 in proliferating epithelial cells of the epidermis and hair follicles, we discovered LMO4 as a Clim2 interacting protein in the epidermis (Sugihara et al., 1998). LMO4, simultaneously discovered by other laboratories (Grutz et al., 1998; Kenny et al., 1998), is the main LIM domain factor expressed in proliferating epithelial cells of the epidermis and hair follicles (Sugihara et al., 1998). Interestingly, the human *LMO4* gene was

initially cloned from a breast cancer cDNA library (Racevskis et al., 1999), and subsequent studies showed it to be overexpressed in more than half of all invasive breast carcinomas (Visvader et al., 2001). Furthermore, LMO4 and Clim2 overexpression interfered with differentiation of cultured mammary epithelial cells (Visvader et al., 2001).

The goals of our studies were to establish a dominant negative LMO4 molecule that can be used to repress transcription of LMO4 target genes and to study the biological function of LMO4 in the mammary gland *in vivo*. We show that fusion of the repression domain from the *Drosophila* Engrailed homeobox protein (Han & Manley, 1993) to LMO4 creates a strong transcriptional repressor, capable of interfering with basal and activated transcription. Expression of this fusion molecule under the MMTV promoter in mammary glands of transgenic mice leads to inhibition of ductular and alveolar development, suggesting that LMO4 is involved in progression of mammary gland development.

LMO4 is up-regulated in mammary epithelial cells during mid-pregnancy and by heregulin in MCF-7 breast cancer cells: To gain insights into the role of LMO4 in mammary gland biology, we assessed its expression in mouse mammary gland and breast cancer cell lines. In contrast to a previous study employing Northern blot analyses on total RNA (Visvader et al., 2001), the sensitive RNAse protection assays show that LMO4 transcripts are easily detected in virgin mammary glands and that expression levels remain relatively stable from age 4 weeks to 14 weeks (Fig. 1a). However, there is dramatic up-regulation of LMO4 in mammary glands from mid-pregnancy, with levels falling late in pregnancy (Fig. 1a), and a moderate increase in LMO4 levels during early lactation (Fig. 1a). Clim2 levels are coordinately regulated during mammary

gland development, with highest levels found in mid-pregnancy (Fig. 1a), consistent with the idea that LMO4 and Clim2 act as a complex. *In situ* hybridization studies on mammary gland sections show that LMO4 is primarily expressed in ductular and alveolar epithelial cells (Fig. 1b). Consistent with the RNAse protection assay experiments, LMO4 levels are high at day 14.5 and lower at day 18.5 (Fig. 1b). The surge in LMO4/Clim2 transcript levels during mid-pregnancy suggests an especially important function at this developmental stage characterized by dramatic epithelial cell proliferation and stromal invasion.

In three different human breast cancer cell lines, LMO4 transcript levels vary from high in the estrogen receptor negative MDA-MB-231, intermediate in the estrogen receptor negative MDA-MB-453, to low in the estrogen receptor positive MCF-7 (Fig. 1c). Estradiol did not increase LMO4 expression in MCF-7 cells (Fig. 1c), consistent with findings in human breast cancer indicating that LMO4 is especially characteristic for estrogen receptor negative tumors (Gruvberger et al., 2001). In contrast to the coordinately regulated expression of LMO4 and Clim2 during normal mammary gland development (Fig. 1a), Clim2 levels remain constant in breast cancer cell lines that express high levels of LMO4 transcripts (Fig. 1c) and protein (Fig. 1d). These results suggest that relative overexpression of LMO4 compared to Clim2 may be important for LMO4 actions in breast cancer. Because LMO4 may be localized to the cytoplasm under certain conditions (Kenny et al., 1998), we evaluated its cellular distribution in breast cancer cells by generating MCF-7 cells stably expressing a myc tagged (MT) LMO4. In these cells, LMO4 is restricted to the nucleus (Fig. 1e). In contrast to the lack of estrogen regulation, LMO4 expression is stimulated by the ErbB ligand heregulin, which is known to be important for alveolar maturation and proliferation (Fig. 1f). Heregulin is thought to act through ErbB2-

containing heterodimers (Stern, 2003) and its effect was partially blocked by an ErbB2 antibody (Fig. 1g), suggesting a role for ErbB2 in heregulin-mediated up-regulation of LMO4.

The observation that LMO4 may be downstream of heregulin/ErbB2 is consistent with findings that the mesenchymally-expressed heregulin α, like LMO4, is strikingly upregulated in mid-pregnancy (Yang et al., 1995). In addition, heregulin and the ErbB2/ErbB3/ErbB4 receptors, which have growth-stimulatory roles (Aguilar et al., 1999; Krane & Leder, 1996), are particularly important for alveolar morphogenesis (Jones et al., 1996; Jones & Stern, 1999; Jones et al., 1999; Li et al., 2002; Yang et al., 1995). ErbB2 is also overexpressed in 15 – 40 % of breast cancer cases where it is associated with increased invasiveness and metastasis, as well as poor prognosis (Eccles, 2001; Slamon et al., 1989). Our findings suggest the possibility that LMO4 may participate in heregulin/ErbB signaling in the mammary gland.

An Engrailed-LMO4 fusion protein is capable of protein-protein interactions and acts as a strong transcriptional repressor: LMO4 forms a complex with Clim co-activators in epithelial cells and is thought to be recruited to DNA-binding proteins, resulting in transcriptional activation of target genes. We hypothesized that fusing the *Drosophila* Engrailed transcriptional repression domain to LMO4 would create a dominant negative molecule capable of suppressing LMO4 target genes (Fig. 2a). When fused to heterologous transcription factors, the Engrailed repression domain confers strong transcriptional repression. This quality was successfully used to obtain insights into the biological function of a spectrum of transcriptionally active molecules, including c-Myb (Taylor et al., 1996), *Xenopus* tailless (Hollemann et al., 1998), GATA factors

(Dasen et al., 1999; Liu et al., 2002; Sykes et al., 1998), homeobox factors iroquois3 (Kudoh & Dawid, 2001) and RaxL (Chen & Cepko, 2002), and β-catenin (Montross et al., 2000).

To test whether the Engrailed-LMO4 fusion protein was capable of protein-protein interactions, we performed co-immunoprecipitation assays in HEK293T cells transfected with expression plasmids encoding tagged LMO4 and Clim2, as well as fusion proteins with LMO4 and Clim2. As expected, Clim2 antiserum precipitated LMO4 in cells transfected with Clim2 and LMO4 (Fig. 2b). In cells co-transfected with tagged VP16-Clim2 and Engrailed-LMO4 fusion proteins, both proteins could be precipitated independent of whether the precipitating antibody was directed against VP16-MT-Clim (Fig. 2c, left panel) or HA-Engrailed-LMO4 (Fig. 2c, right panel). We conclude that the fusion of the *Drosophila* Engrailed repression domain to LMO4 does not interfere with its ability to interact with Clim proteins.

Because natural target genes for LMO4 are unknown, we tested the effectiveness of the Engrailed-LMO4 fusion in a GAL reporter system where we monitored the transcriptional activity of a luciferase reporter gene under the control of GAL DNA-binding sites and a minimal promoter (Sugihara et al., 1998) (Fig. 2d). While LMO4-GAL (Fig. 2d, panel 2) has little effect on basal activity of the promoter, Engrailed-LMO4-GAL (Fig. 2d, panel 3) represses transcription of the reporter gene 29-fold. Furthermore, Engrailed-LMO4-GAL could completely overcome a 105-fold activation conferred by the recruitment of a Clim-VP16 fusion protein (Fig. 2d, compare panels 5 and 6). Clim alone is a weak activator in this system and the Clim-VP16 fusion protein is used because the viral VP16 transactivation domain can confer strong transactivation to heterologous proteins. In summary, these experiments suggest that an

Engrailed-LMO4 fusion protein can repress both basal and activated expression of LMO4 target genes, and that this fusion molecule may be useful to test the biological functions of LMO4.

Expression of the Engrailed-LMO4 fusion protein in mammary gland epithelial cells of mice interferes with mammary gland development: To test the effect of the Engrailed-LMO4 molecule on mammary gland development, we placed it under control of the MMTV promoter (Fig. 3a), which has been extensively used to direct high expression in epithelial cells of mammary glands in transgenic mice (Guy et al., 1992; Kitsberg & Leder, 1996; Krane & Leder, 1996; Muller et al., 1988). Three independent lines expressed the transgene in mammary gland epithelial cells. Expression of the transgene was found both in virgin and pregnant mammary glands (Fig. 3b) and by immunohistochemistry with an HA antibody expression was predominantly nuclear (Fig. 3c). The relatively constant level of the transgene expression (Fig. 3b) is probably because the transgene in this line is upregulated at the very end of pregnancy as has been described for other MMTV transgenic mice (Jones et al., 1999). We examined mammary gland development by whole mount analyses in transgenic mice and compared them to wild-type littermates. Development of transgenic mammary glands of virgin mice was normal at 3 to 4 weeks (data not shown), but at 6 weeks a mild delay in the progression of ductal development was evident (Fig. 3d). At 8 weeks, most transgenic mammary glands were normal (Fig. 3d, lower TG panel at 8 weeks) although we did observe occasional abnormality at that stage (Fig. 3d, upper TG panel at 8 weeks). These data indicate that the Engrailed-LMO4 fusion protein causes a transient delay in mammary gland development of virgin mice.

In pregnant transgenic mice, a clear delay in alveolar development was evident at day 5.5 (Fig. 4a and b); this delay, however, was later overcome and by day 15.5, lobuloalveolar development was essentially normal (Fig. 4b). No abnormalities were observed during lactation (Fig. 4b) and transgenic females were able to nurse normal size litters. In conclusion, expression of the dominant negative Engrailed-LMO4 fusion protein in the mammary glands of mice results in the slowing of ductal development in virgin mice and a transient inhibition of alveolar development during pregnancy, suggesting that LMO4 plays roles in both ductular and alveolar development *in vivo*.

The phenotype of the MMTV-HA-Engrailed-LMO4 mice may be distinct from the expected phenotype of LMO4 null mice. First, the Engrailed-LMO4 fusion protein can suppress the expression of LMO4 target genes both under basal and activated conditions. In contrast, deletion of the LMO4 gene is likely to affect only the genes where LMO4 is actually participating in regulated transcription. Second, while it is generally thought that LMOs in combination with Clims are involved in transactivation, LMO4 may also participate in repression of certain genes as has been suggested with BRCA1-mediated transcriptional activity (Sum et al., 2002). The Engrailed-LMO4 fusion protein would not be expected to affect these genes since they are already repressed. Finally, LMO4 may also act by binding to and sequestering other proteins in solution, a process the Engrailed-LMO4 fusion protein would not be expected to inhibit. Such mechanisms have been proposed for the effect of *Drosophila* lmo in the fly wing (Zeng et al., 1998).

The effect of Engrailed-LMO4 expression in mammary glands was most clearly observed in early pregnancy but the defect was overcome towards the end of pregnancy. Such defects, in which mammary gland development is slowed but not blocked, have been previously described in other genetically modified mice such as those with mutations in the ErbB2 gene (Stern, 2003). However, it is not possible to conclude that the role of LMO4 is restricted to ductular development in virgin mice and alveolar development in early pregnancy because it is impossible to determine which levels of transgene expression are required to block endogenous LMO4 protein levels. Despite these limitations of the dominant negative approach, our results strongly support roles for LMO4 in both ductular and alveolar development. Moreover, the dominant negative LMO4 is a promising tool to evaluate the possible role of LMO4 in signaling pathways and in breast cancer.

The etiology of sporadic breast cancers is multifactorial and thought to involve stepwise mutations in several oncogenes and tumor suppressor genes. The findings described in this paper are of importance because there are parallels between mammary epithelial cells during pregnancy and in breast cancer, and the LMO4 gene is frequently overexpressed in breast cancer. While neoplastic breast epithelial cells clearly have properties distinct from epithelial cells of the developing breast, the two also share similarities such as active proliferation and lack of terminal differentiation (Rudland et al., 1998). Our studies -- showing high expression of LMO4 during a stage in mammary gland development when there is active proliferation and stromal invasion, and the inhibition of these processes with a dominant negative LMO4 molecule -- lend support for the idea that LMO4 up-regulation may contribute to the tumorigenic characteristics of mammary epithelial cells (Visvader et al., 2001).

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Figure legends

Figure 1. Expression of LMO4 and Clim2 during mammary gland development and in response to regulators of mammary gland development. **(a)** RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel) and β-actin (bottom panel) during the indicated stages of mammary gland development. **(b)** *In situ* hybridization study showing expression of LMO4 in mammary glands at day 14.5 of pregnancy (left panel), day 18.5 of pregnancy (middle panel) and day 10 after weaning (right panel). ^{35}S labeled cRNA probes specific for mouse LMO4 were applied to formalin fixed tissue as described (Sugihara et al., 1998). **(c)** RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel) and β-actin (lower panel) in the indicated breast cancer cell lines and with estradiol (E2) treatment (20 ng/ml) for the indicated times. MCF-7 cells were grown in the presence of phenol red-free media and charcoal stripped serum. **(d)** Western blot of whole cell extracts from MDA-MB-231 and MCF-7 cells, using rat LMO4 antibody (Sum et al., 2002) and rabbit Clim antisera (Bach et al., 1999). **(e)** Immunolocalization LMO4 in MCF-7 cells stably expressing myc tagged LMO4. After fixing with formalin, slides were incubated with a myc antibody and signal detected with peroxidase. **(f)** RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel) and β-actin (lower panel) in MCF-7 cells after heregulin treatment with the indicated concentrations for 24 hours. MCF-7 cells were maintained in serum-free media. Similar effects were observed after 48 hour treatment (data not shown). **(g)** RNAse protection assays showing expression of LMO4 (top panel) and β-actin (lower panel) in MCF-7 cells after treatment for 20 hours with heregulin and ErbB2 blocking antibody (Clone Her2-96, Sigma).

RNA isolation and RNAse protection assays were carried out as previously described (Andersen et al., 1997), using ^{32}P-labeled cRNAs specific for mouse and human LMO4, Clim2 and β-actin.

Figure 2. Interactions between Clim2 and LMO4 fusion proteins. **(a)** A model for the activity of the Engrailed-LMO4 fusion protein. Under normal condition (left panel), LMO4/Clim complexes are thought to be recruited to promoters of target genes by associating with DNA-binding proteins (indicated as X), resulting in transactivation. Recruitment of the Engrailed-LMO4 fusion proteins to the same complexes should result in transcriptional repression. **(b)** Immunoprecipitation of cell lysates from HEK293T cells transfected with expression plasmids encoding myc tagged (MT) LMO4 and Clim2, using IgG (lane 1) and Clim antibody (Bach et al., 1999) (lane 2). Western blot was probed with MT antibody (Invitrogen). **(c)** Immunoprecipitation of cell lysates from HEK293T cells (Sugihara et al., 2001) transfected with expression plasmids encoding HA tagged Engrailed-LMO4 and myc tagged VP16-Clim2, using IgG (lanes 1 and 3), MT antibody (lane 2) and HA antibody (lane 4). Western blots were probed with the indicated antibodies. The HA-Engrailed-LMO4 fragment was generated in the mammalian expression vector pCMX by fusing the repression domain representing amino acids 2 – 299 of the Drosophila Engrailed gene (Han & Manley, 1993) to an HA tag at the N-terminus and the full length LMO4 coding sequence at the C-terminus. The pCMXGAL-LMO4 and pCMXGAL-EngrailedLMO4 plasmids contain the full-length LMO4 cDNA and the HA-Engrailed-LMO4 fusion protein linked to the GAL DNA-binding domain. The pCMXVP16-Clim plasmid contains the C-terminal LIM-interaction domain of Clim1 (Bach et al., 1999) linked to the VP16 transactivation domain. **(d)** The indicated GAL DNA-binding domain fusion proteins and VP16 fusion proteins were transfected into HEK293T cells with a GAL-luciferase

reporter plasmid, using calcium-mediated gene transfer (Sugihara et al., 1998). The results, expressed as light units, represent the mean and standard deviation from triplicate transfections. IP, immunoprecipitation; W, Western blot.

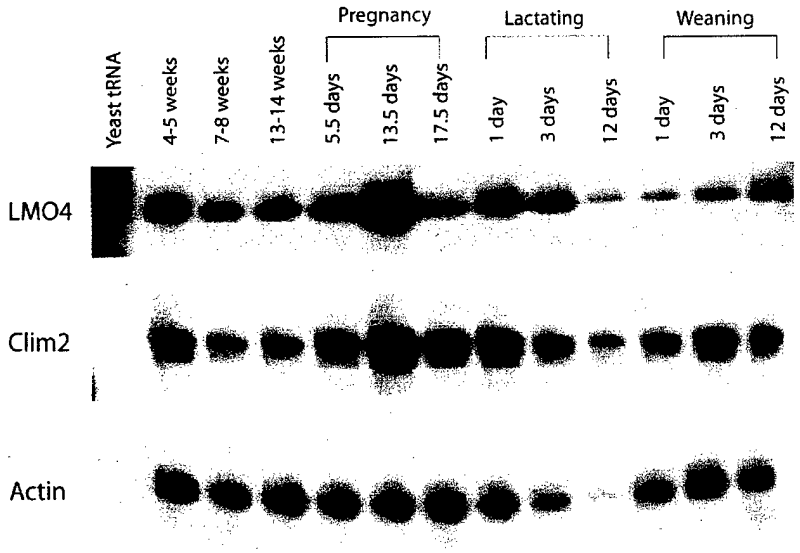
Figure 3. The effect of the Engrailed-LMO4 fusion protein on mammary gland development in virgin mice. **(a)** A schematic of the transgene. The MMTV-HA-Engrailed-LMO4 plasmid was created by cloning the HA-Engrailed-LMO4 fragment into the EcoR1 site of the MMTV-SV40-BSSK plasmid (Leder et al., 1986). To generate transgenic mice, the plasmid was cut with Xho1 and Spe1 to remove extraneous sequences, and the purified DNA fragment was then injected into fertilized CB6F1 oocytes, which were implanted into pseudopregnant mice. Of 13 mice born, five contained the MMTV-HA-Engrailed-LMO4 sequences as assessed by PCR with oligonucleotides specific for MMTV sequences. Of these five lines of founder mice, three (lines #1, 2 and 7) expressed the transgene as assessed by immunohistochemistry with HA antibody on pregnant mammary glands. The three expressing lines were expanded by breeding into CB6F1 mice. Experiments were carried out with transgenic mice derived from lines #1, 2 and 7, which showed a comparable level of abnormality in mammary gland development. **(b)** RNAse protection assays showing expression of the Engrailed-LMO4 transgene from line #7 at the indicated developmental time points. The probe, which corresponded to the *Drosophila* Engrailed part of the fusion molecule, was specific because no signal was observed in mammary glands from wild-type mice (data not shown). **(c)** HA immunostaining of mammary gland (day 1 of lactation) from MMTV-HA-Engrailed-LMO4 mice. Immunostaining of wild-type littermates gave no staining with the HA antibody (data not shown) indicating that the staining is specific. The mammary glands were fixed for one hour at room temperature in a solution composed of 6

parts ethanol, 3 parts water and 1 part formaldehyde, followed by storage in 70% ethanol at 4° C. Paraffin embedded tissue sections were stained with a monoclonal HA antibody (Covance) using peroxidase. **(d)** Whole mount staining of the fourth inguinal mammary glands from MMTV-HA-Engrailed-LMO4 (TG) mice and littermate wild-type (WT) controls at the indicated developmental stages. Shown are representative results from analyses of 16 (6 weeks) and 3 (8 weeks) TG mice. The mammary glands were dissected, processed as a whole mount, fixed and stained with hematoxylin as described (Brisken et al., 1999), and photographed at the same magnification.

Figure 4. The effect of the Engrailed-LMO4 fusion protein on mammary gland development during pregnancy. **(a)** Whole mount overview of mammary glands from 5.5 day pregnant mice comparing wild-type (WT) and transgenic (TG) mice. **(b)** Whole mount analyses in higher magnification from the indicated developmental stages. All magnifications are the same. Shown are representative results from the analyses of 10 (5.5 day), 6 (15.5) and 4 (lactating) transgenic mice.

Fig. 1

a



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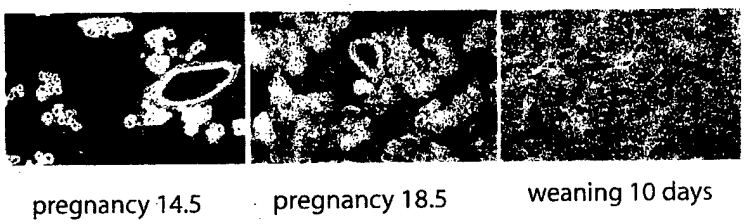

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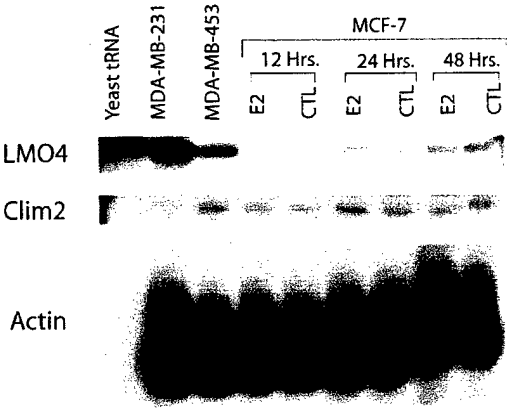

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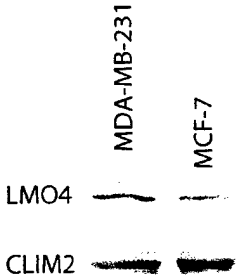
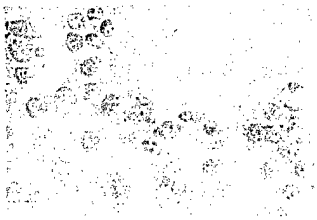


e



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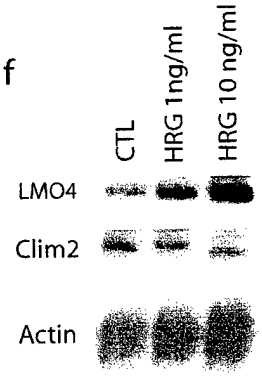

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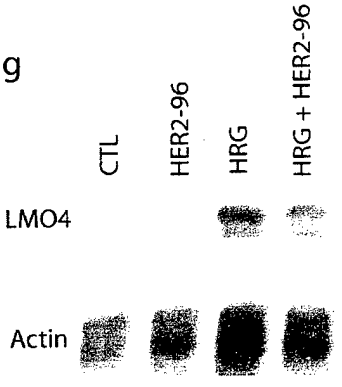
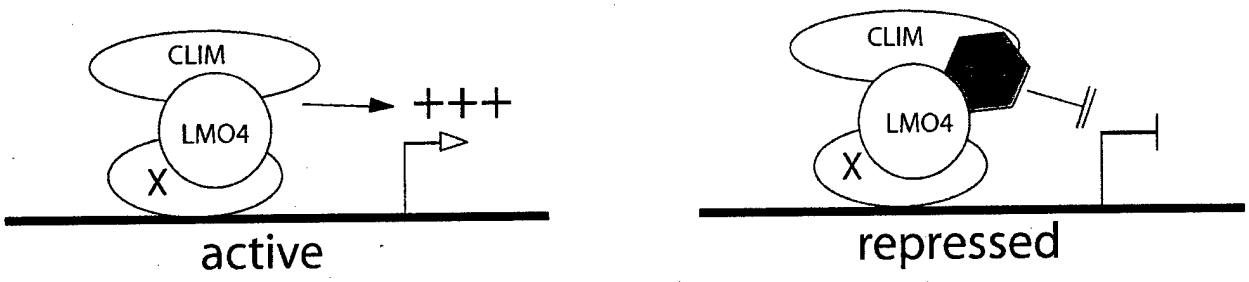


Fig. 2

a



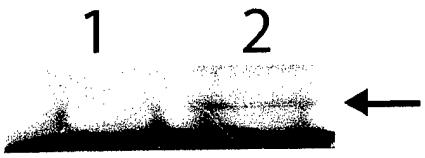
b

pCS2MT-LMO4
pcDNA3-CLIM2

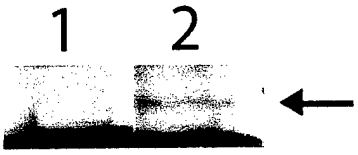
IP: IgG α CLIM2
W: αMT

c

pCMX-VP16-MT-CLIM2
pCMX-HA-Engrailed-LMO4

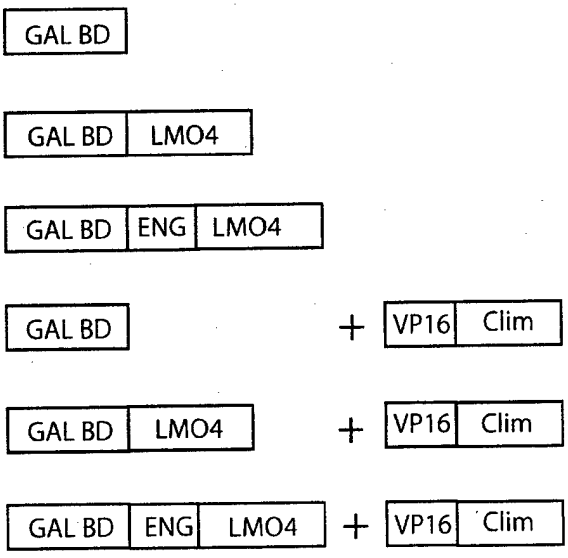


IgG αMT
αHA



IgG αHA
αMT

d



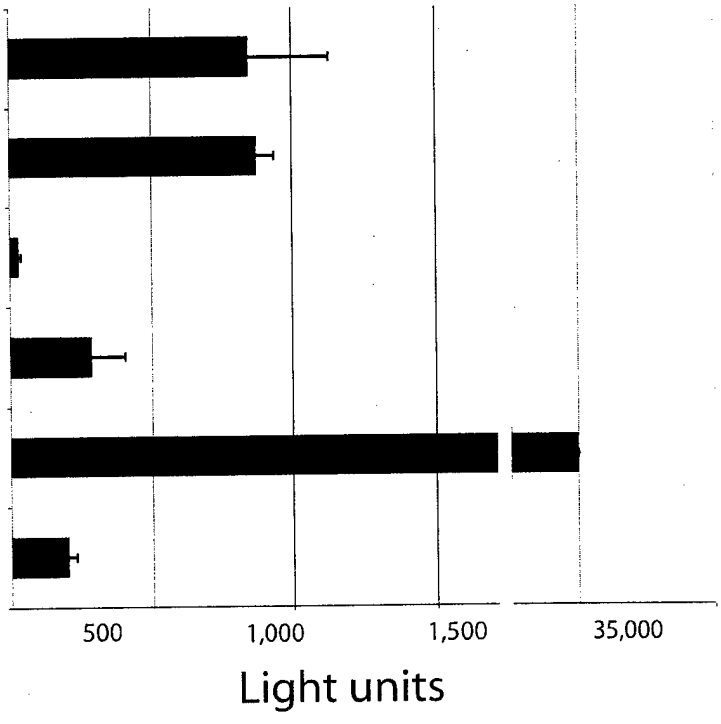
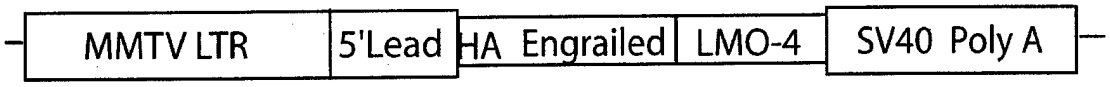
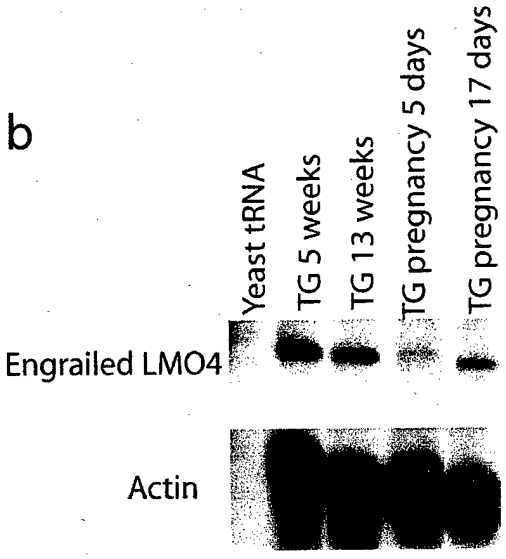


Fig. 3

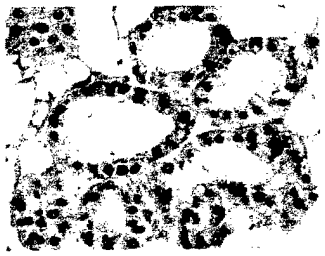
a


MMTV LTR
5'Lead
HA Engrailed
LMO-4
SV40 Poly A

b


Yeast tRNA
TG 5 weeks
TG 13 weeks
TG pregnancy 5 days
TG pregnancy 17 days
Engrailed LMO4
Actin

c



d

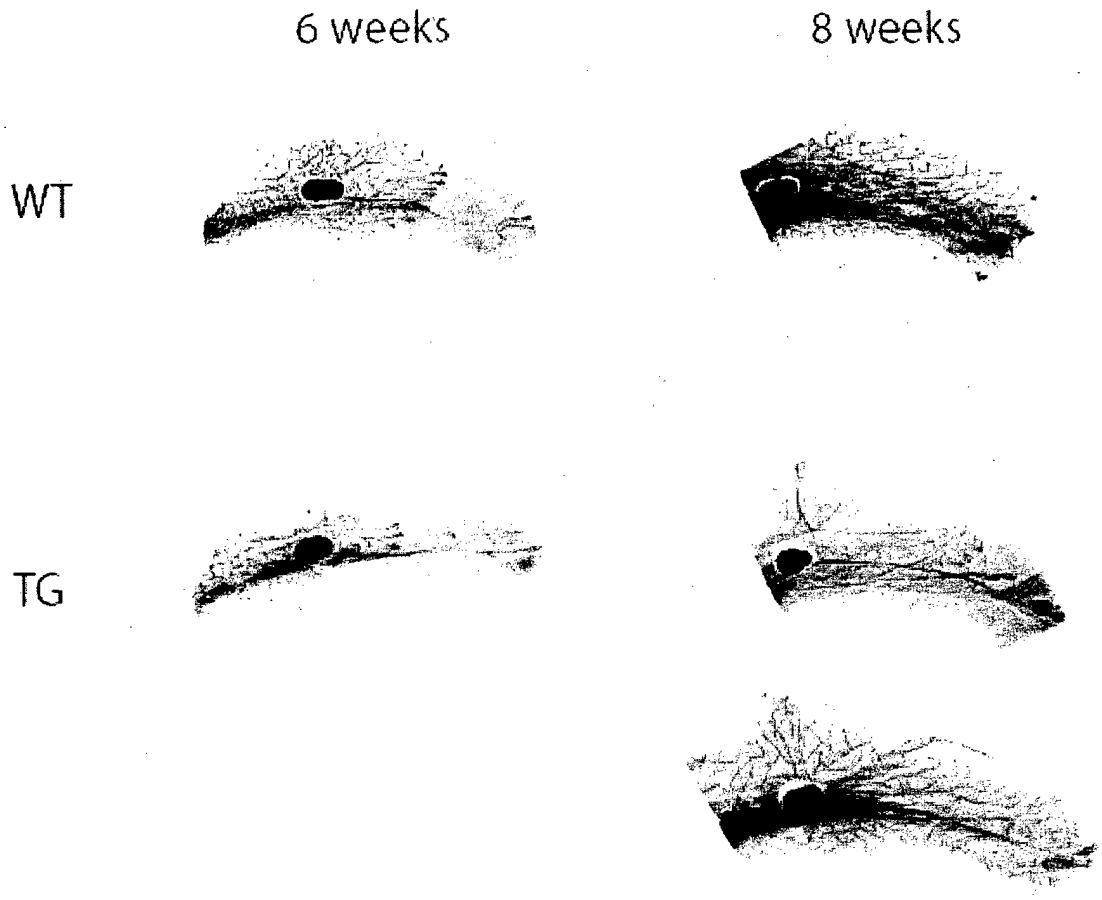
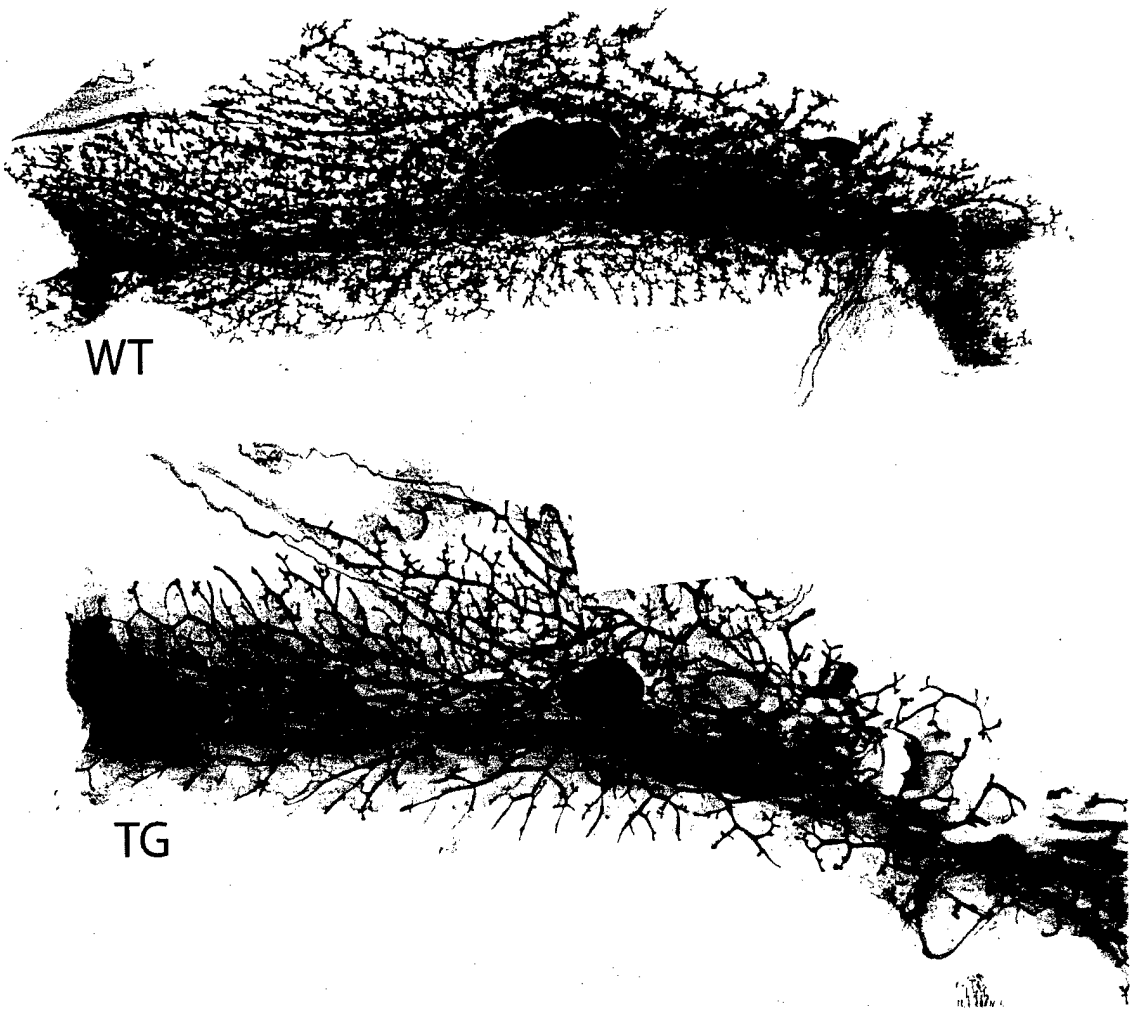

6 weeks
8 weeks
WT
TG

Fig. 4

a



b