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Loss of the LIM domain protein Lmo4 in the mammary gland during pregnancy impedes lobuloalveolar development

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LMO4, a member of the LIM-only family of zinc-finger proteins, is overexpressed in a significant proportion of breast carcinomas and acts as a negative regulator of mammary epithelial differentiation. To delineate cell types within the developing mouse mammary gland that express Lmo4, we analysed different stages of mammopoiesis by immunohistochemistry. Lmo4 was found to be highly expressed in the proliferating cap cells of the terminal end bud and in the ductal and alveolar luminal cells of the mature mammary gland but was negligible or low in myoepithelial cells. To assess the physiological role of Lmo4 in the mammary gland, we generated conditionally targeted mice lacking *Lmo4* in the mammary epithelium during pregnancy. Acute loss of *Lmo4* in late pregnancy impaired lobuloalveolar development, accompanied by a two-fold reduction in the percentage of BrdU-positive cells. In contrast, germline loss of *Lmo4* did not alter lobuloalveolar development arising from transplanted mammary anlagen, implying the existence of a compensatory mechanism in these knockout mice. Thus, the use of a conditional targeting strategy has revealed that Lmo4 is required for proper development of the mammary gland during pregnancy and indicated that Lmo4 acts as a positive regulator of alveolar epithelial proliferation.
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Introduction

Development of the mammary gland involves a complex cycle of morphogenetic changes that are dictated by the coordinated action of growth factors and steroid/peptide hormones. Mammopoiesis commences with ductal elongation and branching in puberty, followed by lobuloalveolar expansion during pregnancy, terminal differentiation of these units in late pregnancy and lactation, and involution of the gland postlactation (reviewed in Hennighausen and Robinson, 1998; Silberstein, 2001; Hovey *et al*., 2002). It is presumed that hormones and cytokines govern these processes by activating or repressing specific combinations of transcription factors and cofactors. Several transcriptional regulators have been established to have critical roles in the developing mammary gland through targeted gene deletion in mice (reviewed in Hennighausen and Robinson, 1998; Visvader and Lindeman, 2003). These include the estrogen receptor α (ERα) and C/EBPβ, which influence ductal outgrowth in the postnatal mammary gland, and Stat5a, the progesterone receptor (PR), as well as C/EBPβ, which regulate lobuloalveolar development during pregnancy.

LMO4 belongs to the LIM-only (LMO) subclass of LIM domain proteins (LMO1–4), each of which is defined by two tandem zinc-finger domains (Dawid *et al*., 1998; Bach, 2000). LMO4 was identified by virtue of its interaction with the ubiquitous cofactor Ldbl/NLI/CLIM2 (Grutz *et al*., 1998; Kenny *et al*., 1998; Sugihara *et al*., 1998) and in an expression screen using autologous serum from a breast cancer patient (Racevskis *et al*., 1999). It is the most distantly related member of the LMO family and, in contrast to the more restricted expression profiles of the other *LMO* genes, is widely expressed in embryonic and adult tissues (Grutz *et al*., 1998; Kenny *et al*., 1998; Sugihara *et al*., 1998; Hermanson *et al*., 1999; Bulchand *et al*., 2003). Like other LMO proteins, LMO4 appears to function as a molecular adaptor for the assembly of multiprotein complexes. LMO2 has been established to form a complex comprising the hematopoietic transcription factors SCL(TAL-1)/E2A and GATA-1 as well as the cofactor Ldbl (Wadman *et al*., 1997). Similarly, LMO4 has been shown to participate in a novel multiprotein complex comprising BRCA1 and CtIP in breast epithelial cells (Sum *et al*., 2002). LMO4 also associates with other proteins, including the transcription factors Deformed Epidermal Autoregulatory Factor-1 /Nuclear Deaf Related factor (DEAF-1/NUDR/Suppressin) (Sugihara *et al*., 1998), Grainyhead-like epithelial transactivator (GET-1)

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(Kudryavtseva *et al*., 2003) and the bHLH protein HEN1 (Manetopoulos *et al*., 2003).

LMO proteins appear to have distinct developmental roles. *LMO2* is essential for both primitive and definitive hematopoiesis, as well as embryonic angiogenesis (Warren *et al*., 1994; Yamada *et al*., 1998, 2000). Compound deletion of *Lmo1* and *Lmo3* in mice causes perinatal lethality, most likely due to a neural defect (Tse *et al*., 2004). Targeted deletion of *Lmo4* has demonstrated the importance of this gene in multiple developmental processes. *Lmo4* mutants die shortly following birth, with 50% exhibiting exencephaly due to failure of neural tube closure (Hahm *et al*., 2004; Tse *et al*., 2004). All mutants show sphenoid bone abnormalities, while cranial nerve defects and homeotic transformations in the rib cage and cervical vertebrae were observed with variable penetrance in several *Lmo4*-deficient mice (Hahm *et al*., 2004). The homeotic transformations suggest that Lmo4 modulates the activity of Hox proteins either by direct interaction or regulation of *Hox* gene expression.

Inappropriate expression of *LMO* genes can lead to oncogenesis. The *LMO1* and *LMO2* genes were originally identified by their translocation in acute T-cell leukemia (Rabbitts, 1998) and are oncogenic in transgenic mice (Fisch *et al*., 1992; McGuire *et al*., 1992; Larson *et al*., 1996; Neale *et al*., 1997). Remarkably, the *LMO2* gene is ectopically activated by retroviral integration in SCID patients who develop T cell leukemia following gene therapy (Hacein-Bey-Abina *et al*., 2003). We have shown that LMO4 is overexpressed in a high proportion of primary breast cancers (Visvader *et al*., 2001), both preinvasive and invasive. More recently, LMO4 has also been found to be overexpressed in carcinomas of the oral cavity (Mizunuma *et al*., 2003). Together these findings underscore the importance of the LMO protein family in regulating normal cell growth and differentiation.

To characterize the precise cell types that express Lmo4 in the developing mammary gland, we performed immunohistochemistry using Lmo4-specific monoclonal antibodies. High levels of Lmo4 were observed in the cap cell layer of terminal end buds in the peripubertal gland and in the ductal and alveolar epithelial cells of the mature mammary gland. To address the physiological role of Lmo4 in the mammary gland *in vivo,* we generated mice deficient in *Lmo4* in the alveolar epithelium that forms during pregnancy, using the *cre-loxP* system. These mice exhibited impaired lobuloalveolar development with a concomitant decrease in cell proliferation. Our findings suggest that Lmo4 acts as a positive regulator of epithelial proliferation in the mammary gland during pregnancy.

Results

Lmo4 expression in the developing mammary gland

Western blot analysis using a rat anti-Lmo4 monoclonal antibody (mAb 20F8; Sum *et al*., 2005) revealed expression of Lmo4 protein at all stages of mammary development (Figure 1). The highest levels were observed in mid- to late-pregnancy, with substantially lower levels evident by day 7 of lactation and throughout involution (Figure 1). This expression profile reflects the *Lmo4* mRNA expression pattern detected by *in situ* hybridization and Northern blot analysis, except that RNA levels were found to decrease earlier than that of Lmo4 protein in late pregnancy (Visvader *et al*., 2001).

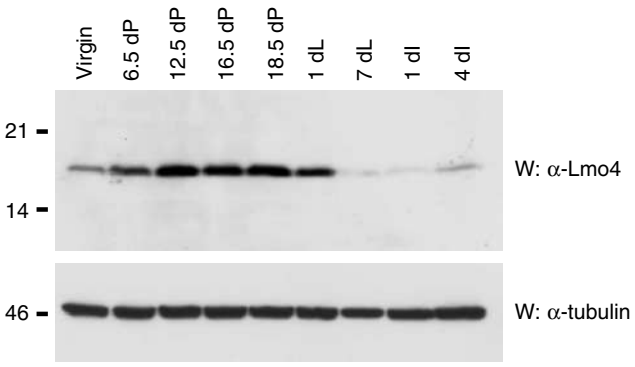

Figure 1 Western blot analysis of Lmo4 expression in the developing mammary gland. Protein lysates (30 μg) prepared from mammary glands of 8-week-old virgin, 6.5-day pregnant (6.5 dP), 12.5-day pregnant (12.5 dP), 16.5-day pregnant (16.5 dP), 18.5-day pregnant (18.5 dP), 1-day lactating (1 dL), 7-day lactating (7 dL), 1-day involuting (1 dI) and 4-day involuting (4 dI) mice were subjected to SDS–PAGE and immunoblot analysis by rat anti-Lmo4 20F8 mAb. Anti-tubulin provided a control for protein loading

Immunohistochemical analysis of Lmo4 expression in the mammary gland using mAb 20F8 revealed prominent staining of Lmo4 in specific epithelial subtypes. Within the primordial mammary bud of the developing embryo at E14.5, low Lmo4 immunostaining was observed, whereas abundant Lmo4 was present in the surrounding mesenchymal cells (Figure 2a). In the mature mammary gland, high levels of Lmo4 were evident in ductal and lobuloalveolar epithelial cells in virgin (Figure 2b), pregnant (Figure 2c–f) and lactating (Figure 2g) mammary glands. Lactation (Figure 2g) and involution (Figure 2h) were accompanied by a decrease in Lmo4 protein levels in the alveolar cells, corresponding to the Western blot data (Figure 1). We also addressed expression in *Lmo4 : LacZ* knockin (KI) mice, in which the *Lmo4* gene promoter drives expression of the *LacZ* gene (Hahm *et al*., 2004). Staining for LacZ activity in mammary glands from these mice at mid-pregnancy confirmed the expression of *Lmo4* transcripts in ductal and alveolar epithelium (Figure 2e). Within the epithelial cells, Lmo4 localized to both the nucleus and cytoplasm. In contrast to the luminal epithelium, Lmo4 expression was low in the surrounding stroma and was undetectable in the majority of myoepithelial cells, as determined by immunohistochemistry (Figure 2b–d and f–h) and LacZ staining (Figure 2e).

We also assessed Lmo4 expression in the terminal end buds (TEBs) of the peripubertal mammary gland (Figure 3). Intense LacZ activity was observed in the cap cells which form the outer monolayer at the tip of

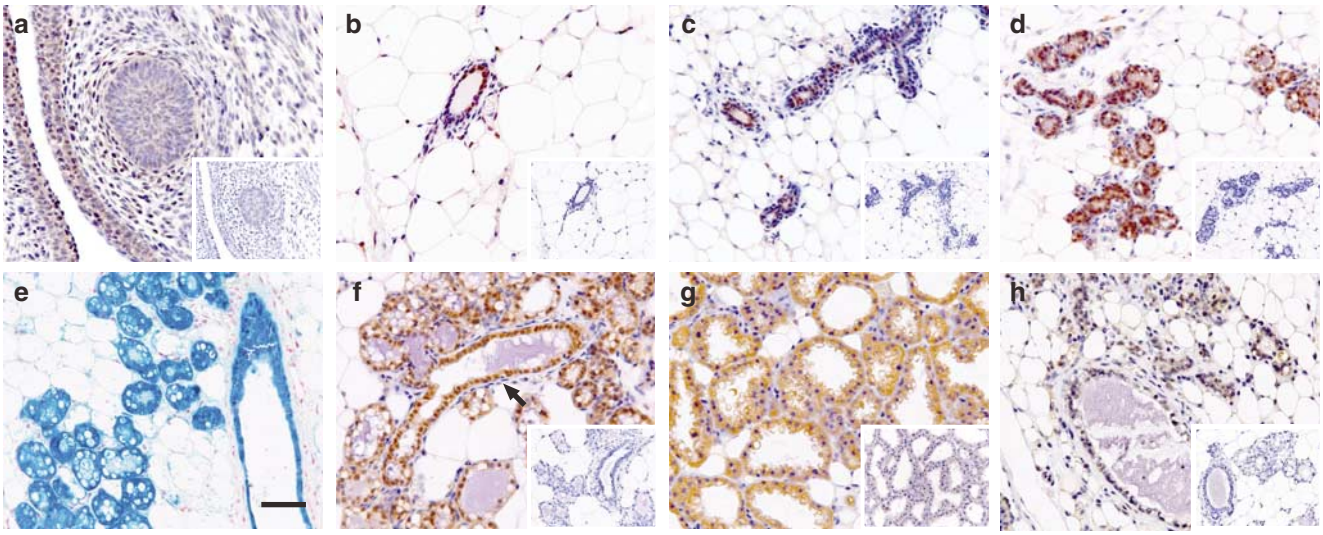

Figure 2 Lmo4 immunostaining of the developing mammary gland: (**a**) Mammary bud (E14.5); (**b**) Virgin adult; (**c**) 6.5-day pregnant; (**d**) 12.5-day pregnant; (**e**) 16.5-day pregnant; (**f**) 18.5 day pregnant; (arrow) myoepithelial cell layer; (**g**) 7-day lactation; (**h**) 4-day involution. Mammary glands were fixed in 4% paraformaldehyde, paraffin embedded and 1.5 μm sections immunostained with rat anti-Lmo4 mAb 20F8 (**a**–**d** and **f**–**h**) or with an isotype-matched non-immune IgG control (insets, **a**–**d** and **f**–**h**). LacZ activity in mammary glands from *Lmo4 : LacZ* knockin mice (**e**) was determined by X-Gal staining of paraformaldehyde-fixed mammary glands, which were then counterstained with nuclear fast red. Panels **a**–**h** are the same magnification. Bar = 50 μm

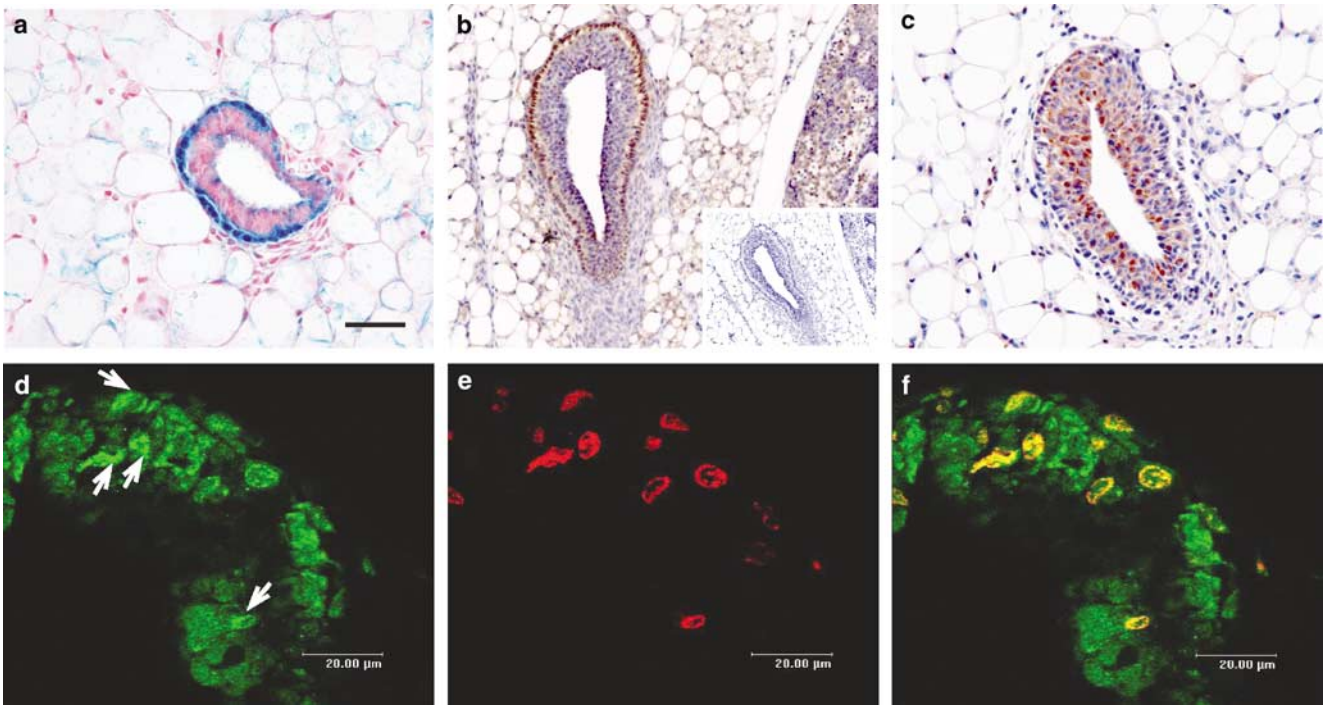

Figure 3 Lmo4 expression in terminal end buds (TEB) of the peri-pubertal mammary gland. (**a**) LacZ activity in a TEB from a 5-week-old female *Lmo4 : LacZ* knockin mouse was determined by X-Gal staining of paraformaldehyde-fixed mammary glands, which were then counterstained with nuclear fast red. (**b** and **c**) Lmo4 and estrogen receptor (ERα) protein expression in TEBs. Mammary glands were fixed in 4% paraformaldehyde, paraffin embedded and 1.5 μm sections immunostained with rat anti-Lmo4 mAb 20F8 (**b**), rabbit anti-ERα mAb (**c**) or with an isotype-matched non-immune IgG control (inset, **b**). (**d**–**f**) Co-localization of Lmo4 and BrdU-labeled cells in the TEB by coimmunofluorescence analysis using confocal microscopy: (**d**) rat anti-Lmo4 20F8 mAb; (**e**) mouse anti-BrdU mAb; (**f**) merged image. Immunofluorescence was visualized using secondary AlexaFluor 488 (green; **d**) goat anti-rat IgG and AlexaFluor 568 (red; **e**) goat anti-mouse IgG antibodies. Arrows indicate cells with the highest Lmo4 levels. (**a**–**c**) and (**d**–**f**) are the same magnification. Bar = 50 μm (**a**) and 20 μm (**d**–**f**)

the bulbous structure that constitutes the TEB, using mammary glands derived from *Lmo4 : LacZ* KI females (Figure 3a). Similarly, immunohistochemistry revealed highest expression of nuclear Lmo4 in these cap cells (Figure 3b), a subset of which are thought to represent stem cells that give rise to both luminal and myoepithelial cells in the mature mammary gland (Williams and Daniel, 1983; Medina and Smith, 1990; Kenney *et al*., 2001). Cells extending beyond the cap region also expressed Lmo4 but at lower levels. Less intense Lmo4 immunostaining was detected in some of the underlying body cells of the TEB, particularly those lining the lumen (Figure 3b). The expression pattern of Lmo4 in the TEB is distinct from that of the estrogen receptor (ERα), which was undetectable in the highly proliferating cap cell layer and appeared to be confined to the body cells (Figure 3c). These data are consistent with previous reports that ERα is not expressed in proliferative cells of the normal mammary gland (Russo *et al*., 1999; Anderson and Clarke, 2004). Moreover, double immunofluorescence staining of TEBs with anti-Lmo4 and anti-BrdU monoclonal antibodies demonstrated that cells with the highest levels of Lmo4 (arrows, Figure 3d) were positive for BrdU (Figure 3d–f), a marker of proliferation.

Mammary glands deficient in Lmo4 exhibit impaired lobuloalveolar development

Lmo4$^{-/-}$ mice die shortly after birth due to complex phenotypic abnormalities (Hahm *et al*., 2004; Tse *et al*., 2004). There were no obvious defects in the mammary buds of *Lmo4*-deficient embryos at E16.5, consistent with the very low level of Lmo4 evident in the mammary primordia of normal mice (Figure 2a). To investigate the physiological role of Lmo4 in the adult mammary gland, we specifically deleted *Lmo4* in the mammary gland using mice carrying a floxed *Lmo4* allele and *WAP-cre* transgenic mice, in which the whey acidic protein (WAP) promoter drives expression of cre-recombinase during late pregnancy. Both *Lmo4*$^{-/fl}$ *: WAP-cre* and *Lmo4*$^{fl/fl}$ *: WAP-cre* mice were generated from mice carrying a floxed *Lmo4* allele and/or a null *Lmo4* allele. Control mice included *Lmo4*$^{-/fl}$ and *Lmo4*$^{fl/fl}$ females that did not harbor the *WAP-cre* transgene and wild-type mice carrying the *WAP-cre* transgene. The mammary glands

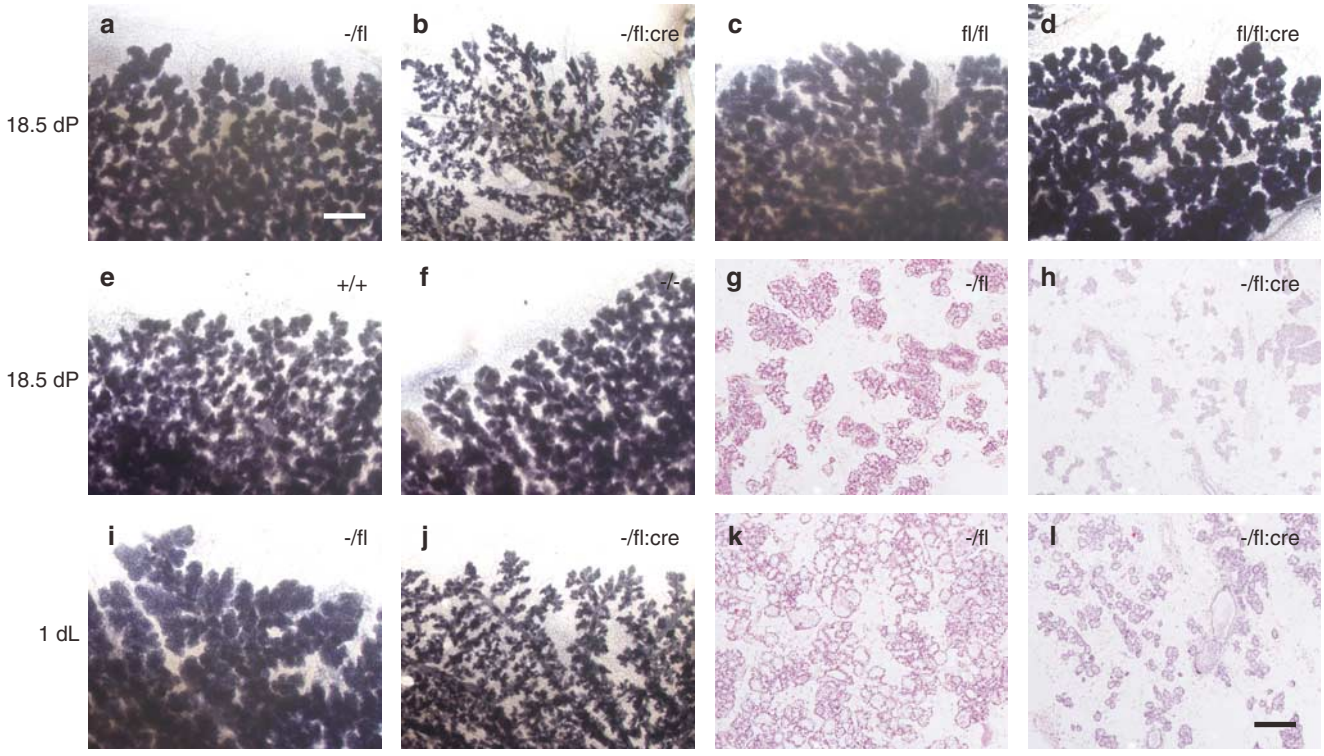

Figure 4 Lobuloalveolar development is impaired in conditionally targeted *Lmo4* mutant mammary glands during late pregnancy and early lactation. Inguinal mammary glands from *Lmo4*$^{-/f}$, *Lmo4*$^{-/fl}$ *: WAP-cre*, *Lmo4*$^{fl/fl}$ and *Lmo4*$^{fl/fl}$ *: WAP-cre* mice at 18.5 dP and 1 dL were excised and prepared for whole-mount analysis by staining with hematoxylin (**a**–**d**, **i**–**j**). For histology, portions of the thoracic and inguinal mammary glands from *Lmo4*$^{-/fl}$ and *Lmo4*$^{-/fl}$ *: WAP-cre* mice were fixed in 10% buffered formalin, paraffin embedded, and stained with hematoxylin and eosin (**g**–**h**, **k**–**l**). *Lmo4*$^{-/fl}$ *: WAP-cre* (**b**, **h**) and *Lmo4*$^{fl/fl}$ *: WAP-cre* (**d**) mammary glands during late pregnancy (day 18.5) revealed reduced lobuloalveolar development (**a**–**d**, **g**, **h**) when compared to control glands (*Lmo4*$^{-/fl}$*:* **a**, **g**; and *Lmo4*$^{fl/fl}$*:* **c**), on both a mixed genetic background and a pure BALB/c background. At day 1 of lactation (**i**–**l**), whole-mounts (**i**, **j**) and histological sections (**k**, **l**) revealed that *Lmo4*^{fl} *: WAP-cre* mice (**j**, **l**) had impaired lobuloalveolar development, in comparison with control mice (*Lmo4*$^{-/fl}$*:* **i**, **k**). Whole-mount analysis of outgrowths from mammary anlage, derived from either wild-type (**e**) and *Lmo4*-nullizygous (**f**) E13.5 embryos, revealed no difference at 18.5 days of pregnancy. The anlage were transplanted into cleared inguinal mammary fat pads of wild-type BALB/c mice. (**a**–**f**, **i**, **j**) and (**g**, **h**, **k**, **l**) are of the same magnification. Bar = 500 μm (**a**) and 200 μm (**l**)

from these control mice were indistinguishable from those of *Lmo4*$^{+/-}$ and wild-type mice (data not shown).

Analysis of whole-mounts of mammary glands and histological sections derived from control and mutant mice revealed that lobuloalveolar development was impaired in *Lmo4*$^{-/fl}$*:WAP-cre* (−/fl:cre) and *Lmo4*$^{fl/fl}$*:WAP-cre* mice (fl/fl:cre) (Figure 4a–d and g–l). A lower density of lobuloalveolar units was observed in *Lmo4*-deficient mammary glands at day 18.5 of pregnancy (Figure 4b, d and h) relative to control glands (−/fl and fl/fl; Figure 4a, c and g). This phenotype was observed on a mixed genetic background and a pure BALB/c background. The extent of cre-mediated recombination in mutant and control mammary glands was estimated by Southern blot analysis (Figure 5a), in which a 10 kb band was generated upon excision accompanied by a decrease in the level of the floxed allele (1.6 kb). Excision was confirmed by Western analysis of corresponding protein lysates from mutant and control mammary glands (Figure 5b and data not shown), with low Lmo4 protein levels correlating with effective excision of the floxed allele, as determined by Southern blotting. Notably, the presence of a phenotype in the mammary gland correlated with the degree of cre-mediated recombination and the resulting level of Lmo4 protein. Of the seven mice exhibiting greater than 50% excision of the *Lmo4* locus and accordingly low Lmo4 expression, five showed markedly decreased lobuloalveolar development relative to control mammary glands (seven mice). As expected, no clear phenotype was apparent in five mice that exhibited poor excision of the locus. Subjecting mice to a second pregnancy was not found to significantly augment the severity of lobuloalveolar impairment (data not shown).

Reduced lobuloalveolar development was also apparent at day 1 of lactation (1 dL) in *Lmo4*$^{-/fl}$*:WAP-cre* mice (Figure 4j and l) relative to control *Lmo4*$^{-/fl}$ mice (Figure 4i and k), as demonstrated by whole-mount and histological analyses. Despite impaired lobuloalveolar development, *Lmo4*$^{-/fl}$*:WAP-cre* females were capable of lactation, as determined by the presence of milk in the stomachs of pups. Two out of six animals analysed at 1 dL revealed substantial excision of the floxed allele in the mammary gland by Southern blot analyses (Figure 5a) accompanied by impaired lobuloalveolar development and low Lmo4 levels (Figure 5b). Extensive histological analyses did not reveal any difference in the size of lipid droplets between mutant and control mammary glands during early lactation.

In addition to the analysis of conditionally targeted mice, we examined mammary epithelial outgrowths derived from transplanted *Lmo4*-null mammary anlagen. Whole-mount analyses of *Lmo4*-null mammary outgrowths in virgin ($n=5$), 5.5 day pregnant ($n=3$) and mid-late pregnant ($n=8$) recipient mice revealed no significant differences compared to internally controlled wild-type or *Lmo4*$^{+/-}$ anlage transplants. Our observations at day 18.5 of pregnancy (Figure 4e and f), in which there is no difference between control and null transplants, contrast with those evident following cell-type specific inactivation of *Lmo4* in the adult mammary gland (Figure 4b and d). There is evidence that somatic versus germline inactivation of the same allele can lead to different phenotypes, suggesting that compensatory mechanisms arise during ontogeny of a conventional

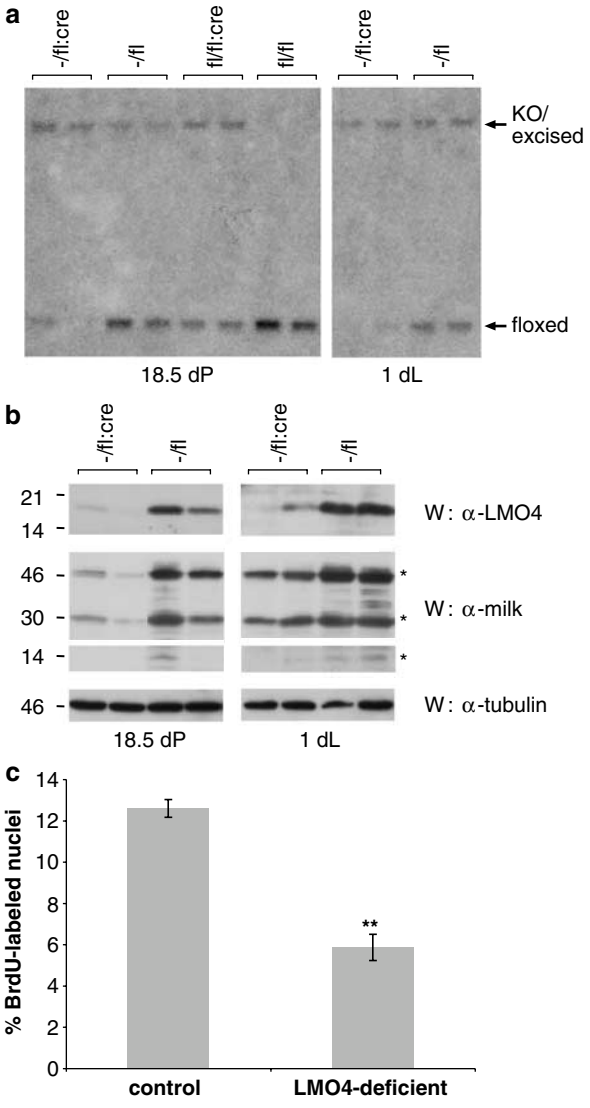

Figure 5 Reduced milk production and proliferation in *Lmo4*-deficient mammary glands. (**a**) Estimation of cre-mediated recombination in mammary glands from *Lmo4*$^{-/fl}$*:WAP-cre* and *Lmo4*$^{fl/fl}$*:WAP-cre* mice compared to control *Lmo4*$^{-/fl}$ and *Lmo4*$^{fl/fl}$ mice by Southern blot analysis. Genomic DNA from the mammary gland was digested with *Xho*I and *Eco*RV, and Southern blot analysis was performed using a 600-bp *Xho*I–*Hind*III probe. Knockout (10 kb), excised (10 kb) and floxed (1.6 kb) alleles are indicated. (**b**) Decreased milk protein in *Lmo4*-deficient mammary glands. Mammary gland protein lysates (30 μg) were analysed by Western blotting using antibodies specific to Lmo4 (20F8) and mouse milk protein (α-casein (46 kDa), β-casein (30 kDa) and WAP (14 kDa) are indicated by asterisks). The filter was reprobed with anti-tubulin to control for loading. (**c**) Quantitation of proliferating cells in control and mutant *Lmo4* mammary glands. The percentage of BrdU-positive epithelial cells in mammary glands from *Lmo4*-deficient mice ($n=3$) was $5.9\pm0.6\%$ *versus* $12.6\pm0.4\%$ in control glands ($n=6$). This was determined by counting greater than 1000 epithelial nuclei in 10 random fields ($\times 400$ magnification) from each mouse. $^{**}P<0.005$ and error bars indicate s.e.m.

knockout mouse (see Discussion). In summary, deletion of *Lmo4* in germline and somatic cells has revealed that this gene participates in lobuloalveolar development during pregnancy but is dispensible for formation and ductal morphogenesis of the mammary gland.

Reduced milk production and proliferation in Lmo4 mutant mammary glands

The condensed acini evident in mammary glands from *Lmo4*$^{-/f}$*:WAP-cre* females at day 18.5 of pregnancy (18.5 dP) and 1 dL suggested that milk production was affected in *Lmo4*-deficient mammary glands. Western analysis of mammary gland lysates using anti-mouse milk antisera revealed lower levels of milk proteins in mammary glands lacking Lmo4 (Figure 5b). Synthesis of α-casein (46 kDa), β-casein (30 kDa) and WAP (14 kDa) was markedly downregulated in glands from *Lmo4*$^{-/f}$*:WAP-cre* females that exhibited substantial excision at 18.5 dP and 1 dL. No changes in the phosphorylation of Stat5 or Erkl/Erk2 were evident in mutant *Lmo4* glands relative to control mammary glands (data not shown).

The decrease in lobuloalveolar development observed in *Lmo4*-deficient mammary glands may reflect aberrant epithelial proliferation since overexpression of Lmo4 in mouse mammary epithelial cells appears to favor the immature state (Visvader *et al*., 2001). The percentage of BrdU-positive epithelial cells in the mammary glands of *Lmo4*-deficient mice at 18.5 dP was found to be $5.9 \pm 0.6\%$, compared to $12.6 \pm 0.4\%$ in control mammary glands (Figure 5c), reflecting a twofold decrease in proliferating cells. In addition, a 1.8-fold decrease in BrdU-labeled cells in mutant *versus* control mammary glands was observed at day 1 of lactation. These results demonstrate that alveolar epithelial proliferation is altered in *Lmo4*-deficient mammary glands and may provide a mechanism underlying the phenotype observed in these mammary glands. Moreover, we have evidence from RNA interference studies that *LMO4* levels regulate cell proliferation in human breast cancer cells (unpublished data).

Discussion

Deregulated expression of LMO proteins is associated with oncogenesis, highlighting the importance of these proteins in governing cell proliferation and maturation. The *LMO1* and *LMO2* genes are oncogenic within T lymphocytes when inappropriately expressed (Rabbitts, 1998; Hacein-Bey-Abina *et al*., 2003) and LMO4 has been shown to be overexpressed in breast cancers (Visvader *et al*., 2001) and squamous cell carcinomas of the oral cavity (Mizunuma *et al*., 2003). Forced expression of *LMO4* in either mammary epithelial or neuroblastoma cells blocks differentiation to milk-producing (Visvader *et al*., 2001) and neurite-forming cells (Vu *et al*., 2003), respectively. These studies suggest that *LMO4* plays a role in regulating cell proliferation and that deregulation of its expression contributes to the development of carcinomas. Here we report that Lmo4 is required for the development of lobuloalveolar units in the mammary gland during pregnancy and that it appears to function as a positive regulator of alveolar proliferation.

High levels of Lmo4 protein occur in diverse epithelial tissues and in cells with a high proliferative capacity (Sum *et al*., 2005). In the peripubertal mammary gland, the cap cells surrounding the TEB express abundant Lmo4. These specialized TEB structures occur at the tips of growing ducts and drive morphogenesis of the mammary gland (reviewed in Silberstein, 2001). TEBs have been demonstrated to have a high proliferative index (Daniel and Silberstein, 1987) and double immunofluorescence staining of BrdU-labeled TEBs revealed that cells expressing the highest levels of Lmo4 were indeed proliferating (Figure 3). Moreover, there was little overlap in Lmo4 and ERα expression, consistent with the notion that steroid hormone receptor expression and proliferation are dissociated in the normal mammary gland (Russo *et al*., 1999; Anderson and Clarke, 2004).

Lmo4, highly expressed in both ductal and alveolar epithelial cells of the mouse mammary gland, plays a role in normal lobuloalveolar development. Specific targeting of the *Lmo4* gene in the mammary gland using a cre-transgene that is activated during late pregnancy led to impaired lobuloalveolar development accompanied by a substantial decrease in milk production. Aberrant alveolar development during early pregnancy (day 5.5) has been reported in transgenic mice expressing an engrailed-Lmo4 fusion protein (Wang *et al*., 2004). However, this defect was transient and development was normal at day 15.5 of pregnancy, in contrast to that observed in mammary glands deficient in *Lmo4*. This difference is likely to reflect the generation of a transgenic fusion protein that acts in a dominant negative fashion *versus* targeted deletion of the *Lmo4* gene itself. Our attempts to address the role of *Lmo4* in early pregnancy using *MMTV-cre* recombinase transgenic mice have been unsuccessful due to ineffective excision of the locus, possibly reflecting chromatin structure. It is noteworthy that Vooijs *et al*. (2001) have previously reported marked differences in *cre*-recombination frequencies between different loci within the same cell.

Although conditional (or acute) loss of *Lmo4* in the alveolar epithelium during pregnancy resulted in impaired lobuloalveolar development, this defect was not apparent in transplantation studies using *Lmo4* knockout tissue. Notably, there is evidence for phenotypic differences occurring between germline *versus* somatic cell deletion or activation of a specific gene. In the mammary gland, while somatic activation of *ErbB2* predisposes mice to mammary tumors, germline activation of the same *ErbB2* allele (knocked into the *ErbB2* locus) renders mice completely resistant to tumors (Andrechek *et al*., 2004). Compensatory mechanisms were proposed to exist in these mice, in which tissues adapt to the expression of a potent oncogene. Moreover, Sage *et al*. reported that acute loss of *Rb* is phenotypi-

cally different from its constitutive absence. Acute loss of *Rb* was shown to stimulate cell cycle re-entry, whereas this did not occur with *Rb*-knockout cells (Sage *et al*., 2003). Similarly, it is possible that *Lmo4*-null cells adapt to the absence of this regulator during development and that they activate compensatory pathways to allow the mammary gland to undergo full lobuloalveolar development. In contrast, cell type-specific inactivation of this gene at a specific developmental time-point (late pregnancy) would not allow time for adaptation and the phenotype becomes manifest.

The defect in lobuloalveolar development evident in *Lmo4* mutant mammary glands may be attributable to decreased cell proliferation. At least twofold less BrdU-positive cells were observed in mammary glands from *Lmo4*-deficient mice relative to that in control glands, in late pregnancy (Figure 5) and early lactation. Since a high rate of proliferation accompanies the formation and expansion of lobuloalveoli during pregnancy, a decrease in the number of proliferating cells would be anticipated to curtail alveolar development. Furthermore, the decrease in milk production is likely to reflect the reduction in epithelial cell number in *Lmo4* mutant mammary glands. Despite a decrease in milk synthesis, females with *Lmo4*-deficient mammary glands were capable of lactation. This finding is reminiscent of that seen in *HRG-α* null mice, which can lactate normally despite pronounced defects in mammary alveolar development (Li *et al*., 2002). Moreover, epithelial proliferation was found to be significantly reduced in these mammary glands (Li *et al*., 2002), paralleling our data for mammary glands deficient in *Lmo4*. The finding that *Lmo4* can influence alveolar proliferation in the developing mammary gland has implications for the role of *LMO4* in breast cancer since increased proliferation resulting from overexpression of this gene may directly contribute to oncogenesis.

Materials and methods

Experimental animals

All experiments with animals were conducted according to the guidelines of our Institutional Animal Ethics Committee. C57BL/6 and BALB/c mice were obtained from our Institute's breeding facility at Kew (Victoria, Australia). The floxed *Lmo4,* null *Lmo4* and *Lmo4:LacZ KI* mice have been described by Hahm *et al*. (2004). Mouse tail DNA was genotyped for wild-type, null or floxed *Lmo4* alleles by Southern blot analysis as described (Hahm *et al*., 2004). *WAP-cre* transgenic mice and their typing by PCR have been described (Wagner *et al*., 1997). Adult female mice were subjected to timed pregnancies which were scored by the observation of vaginal plugs and confirmed by examination of embryos when mammary glands were collected.

Mammary epithelial transplantation

Mammary anlagen transplants were performed as previously described (Robinson *et al*., 2000). Briefly, embryos were taken at E13.5 from intercrosses between *Lmo4* heterozygous mice. Each anlage was placed onto a mesenchymal fragment and cultured for 16–24 h at 37°C in DME-HAM containing 10% FCS, prior to transplantation into cleared fourth mammary fat pads of wild-type BALB/c mice. Each recipient received a *Lmo4*-null transplant in one fat pad, and either a wild-type or *Lmo4* heterozygous transplant in a contralateral fat pad. Whole-mount analyses of mammary epithelial outgrowths were performed at least 6 weeks following transplantation.

Western blotting

Protein lysates from mammary glands were prepared by crushing frozen tissue in liquid nitrogen with a mortar and pestle before transfer into ice-cold KALB lysis buffer (Nicholson *et al*., 1999) supplemented with Complete protease inhibitor tablet (Roche Diagnostics), l0 mM NaF and 1 mM Na_3VO_4. Protein (30 μg) for Western blot analysis was separated on polyacrylamide gels (Novex), before transfer to polyvinylidene difluoride membranes (Millipore). Nonspecific binding of proteins to membranes was blocked by incubation in PBS containing 0.1% Tween-20 and either 5% skim milk or 20% horse serum. The membranes were then probed with rat anti-Lmo4 20F8 mAb (1–2 μg/ml; Sum *et al*., 2005) or rabbit anti-mouse milk antibody (Accurate Chem. Scientific), followed by horseradish peroxidase-coupled secondary antibodies (Amersham Biosciences, Inc.) and developed by ECL (Amersham Biosciences, Inc.). To control for equal protein loading, the blots were probed with anti-tubulin mAb (Sigma).

Immunohistochemistry

For immunohistochemistry, mouse tissues were fixed in 4% paraformaldehyde. Sections were deparaffinized, rehydrated and subjected to antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 20 min. After cooling to room temperature (RT), sections were treated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxide activity, followed by a 30-min incubation in PBS containing 0.1% Triton X-100 (T-PBS) and 10% normal rabbit serum (NRS). Sections were then incubated overnight at 4°C with either anti-Lmo4 20F8 (5–10 μg/ml), anti-ERα MC20 (Santa Cruz), or an isotype-matched control rat antibody (IgG2a/κ,Pharmingen) diluted in T-PBS containing 5% NRS. Staining was detected by incubating with biotinylated secondary antibodies (Dako), followed by HRP-conjugated streptavidin (Dako, LSAB2). Finally, sections were stained with diaminobenzidene (Dako), counterstained with hematoxylin and mounted in DPX (BDH, Poole, UK).

Histology and mammary gland whole mounts

For histological examination of mouse mammary glands, tissues were fixed in 10% (v/v) formalin in PBS and embedded in paraffin. Sections (1.5 μm) were prepared and stained with hematoxylin and eosin (H&E). For whole-mount analysis, mammary glands were harvested from 18.5 dP and 1 dL mice. Mammary tissues were fixed in Carnoy's solution (six parts 100% ethanol, three parts $CHC1_3$, one part glacial acetic acid) and stained with hematoxylin.

Whole-mount staining for β-galactosidase activity

Mammary tissue from *Lmo4 : LacZ* KI mice was fixed in PBS containing 2% paraformaldehyde and 0.25% glutaraldehyde for 2 h at 4°C prior to staining for β-galactosidase activity by incubation with X-gal solution (5 mM K_3Fe, 5 mM K_4Fe, 2 mM $MgCl_2$, 0.02% NP40 in PBS, supplemented with 0.5 mg/ml X-gal) at 37°C overnight. These tissues were then postfixed in 4% paraformaldehyde overnight at 4°C and embedded in paraffin.

Sections (7 μm) were prepared and counterstained with nuclear fast red.

Bromodeoxyuridine (BrdU) immunodetection

Mice were injected with bromodeoxyuridine (BrdU) Cell Labelling Reagent (0.5 mg/10 g body weight, Amersham Biosciences) 1 h prior to tissue collection. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. For immunohistochemical detection of BrdU-labeled cells, rat anti-BrdU (Becton-Dickinson) and biotinylated rabbit anti-rat IgG antibody (Dako) were used, followed by HRP-conjugated streptavidin (Dako, LSAB2). The percentage of BrdU-labeled nuclei was determined by counting greater than 1000 epithelial nuclei in 10 random fields ($\times$400 magnification) from each mouse.

For Lmo4/BrdU double immunofluorescence staining of terminal end buds (TEBs), mammary gland sections from 5-week-old mice were deparaffinized, rehydrated and subjected to antigen retrieval as described in the previous section. A 30-min incubation in 0.2 M glycine was followed by blocking in PBS containing 0.5% Tween-20 and 5% normal goat serum for 4 h. Sections were then incubated overnight at 4°C with anti-Lmo4 rat monoclonal antibody 20F8 (10 μg/ml) and anti-BrdU mouse monoclonal antibody (clone BU-1; Amersham Biosciences). Staining was detected with secondary AlexaFluor 488 (green) goat anti-rat IgG and AlexaFluor 568 (red) goat anti-mouse IgG antibodies (Molecular Probes). Finally, sections were washed in PBS, mounted in Fluorescent Mounting Medium (Dako) and visualized by confocal microscopy (Leica TCS.NT.SP2).

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