Title Page
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PML depletion disrupts normal mammary gland development and skews the composition of the mammary luminal cell progenitor pool

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Abbreviations: PML, Promyelocytic leukemia; ND, nuclear domain; Stat, signal transducer and activator; MEF, mouse embryonic fibroblast; IFN, interferon; MGD, mammary gland development; JAK, Janus kinase; ER, Estrogen receptor

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Abstract

Nuclear domains of promyelocytic leukemia protein (PML) are known to act as signaling nodes in many cellular processes. Whilst the impact of PML expression in driving cell fate decisions for injured cells is well established, the function of PML in the context of tissue development is less well understood. Here, the *in vivo* role of PML in developmental processes in the murine mammary gland has been investigated. Data is presented showing that expression of PML is tightly regulated by sequential activation of different members of the Stat family of transcription factors that orchestrate the development of the mammary epithelium. A developmental phenotype was discovered in the PML *null* mouse, typified by aberrant differentiation of mammary epithelia with abnormal ductal and alveolar development. PML depletion was also found to disturb the balance of two distinct luminal progenitor populations. Overall, it is revealed that PML is required for cell lineage determination in bi-potent luminal progenitor cells and that the precise regulation of PML expression is required for functional differentiation of alveolar cells.

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Introduction

The promyelocytic leukemia protein (PML) is a tumor and growth suppressor and pleiotropic regulator of multiple cellular processes including cell death, differentiation, and stress response pathways (1-3). PML resides in large macromolecular protein complexes known as PML nuclear domains PML-NDs (PML bodies/ND10/POD) of which it is an essential component (2). The role of PML-NDs is enigmatic (1, 3) but their presence is vital for the correct function of PML protein as evidenced by PML's role in acute promyelocytic leukemia (APL) and by mouse models of PML loss. The majority of acute promyelocytic leukemia (APL) cases involve the t(15;17) chromosomal translocation that results in a genetic fusion of the *PML* gene with that of the retinoic acid receptor alpha (*RARα*) (4). The resulting fusion protein, PML-RARα, acts as dominant negative mutant of both proteins leading to loss of PML-NDs, a block in myeloid cell differentiation and subsequent leukaemogenesis (4, 5). Loss of PML in mouse models leads to a resistance to apoptosis, increased cellular growth rates and a higher sensitivity to a range of tumour-inducing treatments and mutations, underlining the importance of PML in a host of physiological processes (6, 7). However, the PML null mice are conspicuous for their lack of developmental phenotypes other than a mild general anemia (7), although recent data has pointed to a role for PML in preserving tissue stem cell populations (8).

PML expression has been widely studied in both normal and neoplastic tissues (9-15). Expression is widespread in normal tissues, but, in general (and outside of the stem cell compartment), high levels of PML are associated with an inflammatory cytokine environment (9) and regions of high cellular proliferation (9), although some evidence disputes this (11). PML is also down-regulated during differentiation in several tissues such as the myeloid lineage and several epithelia (8, 11, 12, 16). PML expression is disregulated in many types of neoplasia (10, 13) consistent with its roles in regulation of cell growth, fate and differentiation. Despite the extensive studies of PML protein expression, there exists little knowledge of the molecular mechanisms that underlie its

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transcriptional regulation. *Pml* is, however, a well characterized interferon response gene (17, 18) with promoter elements (both ISRE and GAS sites) that function as binding sites for the signal transducer and activator of transcription (Stat) family of transcriptional regulators (19). Hence *Pml*'s up-regulation in an inflammatory cytokine environment can be explained by classical activation of the Janus kinase (JAK)/Stat signaling pathway by interferons. Additionally, the inhibitory action of PML on Stat1α activity (activated by IFN-gamma) in MEFs reveals a complex pattern of PML induction and negative feedback (20).

The Stats act as key transcription factors in response to cytokine signaling thereby modulating many survival, growth and differentiation pathways. There exist seven Stat proteins, some of which interact as heterodimers. In the classic signaling pathway, receptor-associated JAK proteins are phosphorylated in response to cytokine stimulation and then tyrosine phosphorylate Stat proteins that are recruited to the cytokine receptors whereupon they dimerize, translocate to the nucleus and directly activate transcription. The Stat proteins play a key role in the development of the mammary gland (21), a tissue that provides an excellent model of differentiation, death and remodeling. The mammary gland epithelium is set in an adipocyte-rich stroma (known as the fat pad) and undergoes extensive proliferation and differentiation in response to steroid hormones. During puberty, ductal epithelial cells (luminal and myoepithelial) proliferate to form a branched ductal network. At the onset of pregnancy, the epithelial cells begin to proliferate again in response to progesterone, resulting in tertiary branching and formation of lobuloalveolar structures that are the site of milk production. Finally, following weaning, the mammary gland undergoes a period of extensive cell death and remodeling known as involution. These distinct phases of development are tightly controlled by a delicate balance of hormones and cytokines. Further, it is the hierarchy of expression of the Stat proteins that helps to govern the responses of the mammary gland to such developmental signals (21, 22). Hence, Stat6 loss is associated with a delay in alveolar development during pregnancy (23), prolactin-mediated Stat5 activity stimulates functional lobuloalveolar development during pregnancy and milk production during lactation (24) while LIF induces Stat3 activity at the onset of involution to initiate the death and re-modeling

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processes (25). In this study it is shown that interplay between PML and Stats 3, 5 and 6 helps to control the normal development of the mammary gland. PML loss results in abnormal ductal development and differentiation of the mammary epithelium and is surprisingly a regulator of branching morphogenesis. Finally, it is demonstrated that PML loss is also associated with an imbalance in the populations of progenitor cells that contribute to the different luminal epithelial lineages in the gland.

Results

PML is transcriptionally regulated during mammary gland development

The expression of PML protein was found to be tightly regulated, at both the transcript and protein level, during the distinct phases of mammary gland development (MGD) (Fig. 1). The virgin gland was found to contain relatively high levels of PML protein as analyzed by immunoblotting of whole gland preparations (Fig. 1A) and immunohistochemistry of murine tissue sections (Fig. 1B). Expression was found in all cell types but was especially prominent in the epithelium (data not shown). However, during gestation and lactation the amount of PML protein declined to undetectable levels and after 10 days of lactation (10L) PML-NDs were no longer observed in the epithelium (Fig. 1B), although they remained present in other cell types. Since immunoblotting was carried out on whole glands which contain a mixture of epithelia, fibroblasts and adipocytes, the level of PML was compared to that of the epithelial marker E-cadherin (Fig. 1A). From this data, it is clear that the levels of PML in the mammary epithelium are highest in the virgin gland, when the PML-NDs are large and numerous, and decline during lactation, with no PML-NDs detectable at 10L. Remarkably, during the initial stages of involution the level of PML protein increases as evidenced by the re-forming of small micro-bodies of PML (Fig. 1B) that eventually nucleate many, large PML-NDs at later time-points.

Examination of the levels of PML mRNA in the same tissues indicated transcriptional regulation of PML in keeping with the pattern of protein changes (Fig. 1C). Transcripts corresponding to both known splice variants of murine PML were tested and found to be similarly regulated although the differential expression of either transcript was not as marked as that seen at the protein level. This suggests that at the protein level, the two PML isoforms may be differentially regulated. Protein levels of the smaller isoform (isoform 2 – lower band in Fig. 1A) were higher in the virgin gland, whereas the larger isoform (isoform 1 – upper band in Fig. 1A) accumulated during the course of development and was noticeably higher at day 5 lactation.

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PML loss has been associated with the differentiation of specific cell types, particularly those of the hematopoietic and epithelial compartments (8, 11, 12, 16). The suppression of PML during the gestation and lactation stages of mammary gland development was therefore postulated to be associated with the differentiation of the mammary epithelium. To test this, the conditionally immortal murine mammary epithelial cell line KIM-2 was tested for PML expression during a time-course of treatment with prolactin, and dexamethasone. This lactogenic hormone cocktail results in the differentiation of these cells in a manner which mimics the *in vivo* progression of the mammary epithelium from virgin through gestation to lactation (26) and, indeed, PML expression was found to reduce over the course of this treatment (Fig. 1D). Notably, expression of PML isoform 2 was almost completely lost by day 10 of the KIM-2 differentiation time course, a stage that approximates mid-lactation, consistent with the *in vivo* results.

PML expression is regulated by multiple Stat activities

The Stat proteins are key regulators of mammary gland development and several members of this family of transcription factors have been implicated in multiple aspects of this process (23, 24, 25). The presence of GAS and ISRE sites in the PML promoter suggested that the Stat proteins may be responsible for the regulation of PML expression *in vivo*. In order to provide clues as to which Stats may be responsible for such regulation, Stats were focused on that have been shown previously, using gene deletion studies, to have critical roles in normal mammary gland development during a pregnancy/lactation/involution cycle. Stat6 is important in early gestation in promoting proliferation and development of lobuloalveolar cells (23) while Stat5 is an essential mediator of prolactin signaling and is required for functional differentiation of lobuloalveoli and for milk secretion (24). Stat3 is specifically activated at high levels at the onset of post-lactational regression (involution) when it is critical for this process (27). Taken together these data suggest that the balance of multiple Stat activities may affect the level of PML expression during MGD.

Stat3 and Stat6 repress PML expression

The role of Stat proteins in regulating PML expression was analyzed further using both *in vivo* and *in vitro* models. Microarray analysis of mammary glands at 24 hours involution in which *Stat3* had been conditionally deleted showed that the *Pml* gene was up-regulated in comparison to wild type glands. Quantitative RT-PCR analysis of RNA extracted from the same glands confirmed that *Pml* transcription was increased by up to 8-fold in the *Stat3* null glands (Fig. 2A). This transcriptional increase was reflected by an increase in PML protein level and by an increase in the number and intensity of PML-NDs in the epithelium (Fig. 2A). Furthermore, treatment of the mammary epithelial cell line, EpH4, with oncostatin M (OSM), a direct and strong inducer of Stat3 activity, down-regulated PML expression *in vitro* in as little as 2 hours as measured by immunoblot and immunofluorescence (Fig 2B).

Similar results were obtained in studying the interplay between PML and Stat6. At day 15 of gestation in *Stat6* null mouse mammary glands, PML levels were found to be up-regulated with respect to the wild type glands and the number and intensity of PML-NDs in the epithelium was increased (Fig. 2C). Concordantly, Stat6 activation in EpH4 cells by IL-13 led to down-regulation of PML protein levels (Fig. 2D). Although the interplay between Stat5 and PML levels *in vivo* was not assessed, differentiation of KIM-2 cells in response to prolactin severely diminished PML expression levels (Fig. 1D). It is clear, then, that the balance of Stat activities *in vivo* results in the overall regulation of PML protein levels in the mammary epithelium.

PML regulates ductal and branching morphogenesis *in vivo*

Based on the hypothesis that PML may influence MGD, mice lacking the *Pml* gene were analyzed at different stages of a developmental cycle. Considering that mammary ductal morphogenesis occurs primarily during adolescent development and that PML is a known growth suppressor (7), glands from 6 week virgin Pml$^{-/-}$ mice were analyzed for their architectural characteristics and compared to heterozygous littermates. Whole gland

mounts and hematoxylin and eosin (H&E)-stained sections from mice lacking PML were found to have significantly smaller ducts with reduced numbers of branches per duct (Fig. 3A). Quantitative analysis of these data indicated that lumen size in ducts from Pml$^{-/-}$ mammary glands was reduced by an average of 65% whilst the number of branch points per duct was reduced by 40%. These data uncover a surprising role for the PML protein in regulation of *in vivo* ductal morphogenesis. Further analysis of these glands by immunohistochemical staining for markers of the ducto-luminal (cytokeratin 18) and ducto-basal (p63 and smooth muscle actin) epithelial cells showed that, whilst the ducts were globally smaller, the cellular organization remained consistent (Supplementary Fig 1). During gestation a substantial expansion of the mammary epithelium takes place in order to generate the milk-producing alveoli. Glands from PML$^{-/-}$ mice at 15 days gestation were analyzed and found to contain reduced levels of both phosphorylated Stat5 and secreted beta-casein (Fig. 3B), two functional markers of alveolar development.

PML influences mammary epithelial differentiation by altering progenitor populations

In order to probe the possible causes of PML's disruption of mammary morphogenesis a comparative transcriptome analysis was carried out on virgin glands from heterozygous and Pml null mice. Despite a conspicuous lack of up-regulated mRNA's found in the Pml$^{-/-}$ glands, a number of transcripts were found to be significantly down-regulated. Remarkably almost all down-regulated transcripts corresponded to genes associated with epithelial differentiation including whey acidic protein (WAP) along with several caseins and cytokeratins (Table 1) (28, 29) suggesting that PML disrupts the development of the mammary gland by inhibiting epithelial differentiation.

Recently, mammary gland differentiation pathways have begun to be delineated with the identification of discreet populations of progenitor cells which are capable of growing *in vitro*, repopulating cleared mammary fat pads and specifically differentiating into subsets of functionally different epithelial cells (30, 31). Luminal epithelial cells can be divided

into differentiated (CD24hi CD49b$^-$) and progenitor (CD24hi CD49$^+$) compartments based on their ability to form colonies *in vitro* (Stingl and Watson, manuscript in preparation). The progenitor compartment can be subdivided further based on Sca1 staining into Sca1$^-$ (ERα^- luminal progenitors) and Sca1$^+$ (ERα^+ luminal progenitors) populations (Supplementary Fig. 2). In this study the Sca1$^-$ and Sca1$^+$ progenitor populations from the PML$^{+/-}$ and PML$^{-/-}$ mammary glands were analyzed. The Sca1$^-$ luminal progenitors were significantly reduced in PML$^{-/-}$ mice (20 ± 1.9%) compared to PML$^{+/-}$ mice (53 ± 4.6%) (Fig. 4A). In contrast there was an increase in the number of Sca1$^+$ cells in PML$^{-/-}$ mice (57 ± 14%) compared to PML$^{+/-}$ mice (30 ± 2%) (Fig. 4A). These populations were sorted and analyzed for their cloning efficiency. Surprisingly the cloning efficiency of unsorted (total) epithelial cells was significantly greater in PML$^{-/-}$ compared to PML$^{+/-}$ mice (Fig. 4B). Epithelia from heterozygotes generated 4.4 (+/- 0.35) colonies per 100 cells whilst the knock-out animal generated 6.6 colonies (+/- 0.2) $p<0.02$. This increase is most likely due to the increase in the cloning efficiency of the Sca1$^+$ luminal progenitors (Fig. 4B). Consistent with these results, immunostaining for estrogen receptor (ERα) revealed a marked increase in the number of positive cells in Pml$^{-/-}$ glands compared to Pml$^{+/-}$ (Fig. 4C and D). Interestingly, although there is a reduction in the number of Sca1$^-$ luminal progenitors, PML deficiency did not affect the cloning efficiency of this reduced population (Fig. 4B). This data indicates that PML regulates both the ability of mammary progenitors to proliferate and the balance of specific progenitor populations. Loss of PML therefore affects mammary differentiation by favoring a more primitive progenitor type and by skewing the relative populations of specific epithelial lineages.

Discussion

In this study a physiological function for the PML protein in the development of the mammary gland is presented. Throughout the course of mammary gland development there exist phases of significant cellular growth, differentiation and death. Here it is shown that PML's function in this tissue is to regulate the balance of progenitor cell populations that contribute to the growth and expansion of the mammary epithelium. Previous studies of PML have focused on its role in stress and innate immune responses as well as its role in tumour suppression (1, 3, 18). However, a recent report of a physiological role for PML in regulating hematopoietic stem cell maintenance (8) suggests that further developmental functions may exist. In this study PML has been shown to impact mammary epithelial differentiation by regulating progenitor cell populations with the loss of PML resulting in deregulated ductal and alveolar development.

Mammary gland development is regulated by cytokine and hormonal signals and different members of the Stat family are required throughout this process, as shown by genetic ablation studies *in vivo* (21-23). The initial observation that PML expression is highly regulated during MGD was followed by the discovery that Stat3 and Stat6 signaling negatively regulates PML expression in this tissue and this is in keeping with previous evidence that PML is a direct Stat1/2 target (20, 32). PML expression was found to be highest in the virgin mammary gland and at its lowest during lactation. It can be postulated that reduced levels of PML protects the alveolar cells from dying during a time when they are producing extra-ordinary amounts of milk protein. During involution, PML expression was re-induced, resulting in the expression of small 'micro-bodies' of PML which, over time, developed into larger, classical PML-NDs. This re-induction of PML-NDs coincides with massive tissue reorganization and cell death. It is clear, then, that a combination of cytokine and growth factor signaling through the Stat family of signal transducers tunes the levels of PML expression to regulate normal MGD.

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Analysis of *Pml* null mice revealed a novel function in the regulation of branching morphogenesis indicated by aberrant duct size and branch numbers in virgin PML$^{-/-}$ mammary glands. Both microarray studies of virgin glands and observations of reduced Stat5 activity and beta-casein expression during gestation in these mice indicated that dis-regulated epithelial differentiation was the functional cause of PML's role in these developmental processes. A further major finding in this study was the identification of skewed mammary gland progenitor cell populations in PML depleted animals. Recent data has highlighted a role for PML in preserving quiescent stem cell niches (8). In this scenario, high levels of PML were used to maintain quiescence. Here, altered distributions of progenitor cells are described, cell types which are often described to be exquisitely sensitive to stress and cell death. Our demonstration of enhanced replication of progenitors after a week of cell culture is consistent with stem cell data (8) in which exhaustive cell cycling initially generates elevated levels of stem cells which are depleted at later time-points. Indeed, alveolar progenitor numbers are depleted in 14 week virgin mice though no difference was found in 8 week mice.

The physiological roles of PML protein are wide-ranging, encompassing a multitude of cellular signaling pathways and heterogeneous protein complexes coordinated by PML. These dynamic interactions combined with alterations of PML-ND resident 'cargo' proteins in different cell types and in different cellular contexts (such as the local cytokine environment) could underlie PML's pleiotrophic functions. Future work to deconstruct the signaling downstream of PML that allows it to control epithelial differentiation will add to our increasing knowledge of the complex regulatory processes that govern ductal development and improve our understanding of the precise physiological role of the PML protein.

Materials and Methods

Mice

Pml^{-/-} animals backcrossed to the sv129 S2 strain were a kind gift of Dr Paolo Salomoni. *Stat6*^{-/-} (23) and BLG-Cre/*Stat3*[flox+/-] conditional knockout (27, 33) mice are previously described. All animals were maintained in the Biological Services facility of the Department of Pathology at the University of Cambridge and were bred and subjected to listed procedures under the UK Home Office guidelines.

Mammary gland preparations

For whole mount and hematoxylin and eosin analysis glands were prepared as previously described (23).

RNA preparation and PCR analysis

RNA was extracted and quantitative real-time detection of cDNA was performed as previously described (23). Detection of PML was performed using the following primers: Isoform 1: Forward, CCAGAGGAACCCTCCGAAGA; reverse GCTCTCGAAGAACACGTTATCC; Isoform 2: Forward, CAGGCCCTAGAGCTGTCTAAG; reverse ATACACTGGTACAGGGTGTGC.

Transcriptome analysis

cDNA from 4 week old virgin *PML*^{-/-} mice was compared to PML^{+/-} cDNA (n=3) via hybridization to Illumina Mouse-6 Expression BeadChips at the Pathology Department Centre for Microarray Resources, Cambridge, UK.

Cell Culture

EPH4 cells were maintained in 1:1 DMEM:F12 medium supplemented with 10% FCS (v/v). KIM-2 cells (26) were grown to confluency in 1:1 DMEM:F12 (Invitrogen) media containing 10% FCS (Sigma), 0.8 mM Insulin (Sigma), 0.8 mM EGF (Sigma) and 17 mM Linoleic acid (Sigma). For differentiation studies, cells were grown to confluency before addition of differentiation media – comprising 1:1 DMEM:F12, 10% FCS, 0.8

mM insulin, 0.2 mM prolactin (Sigma), 1 mM dexamethasone (Sigma) and 17 mM linoleic acid. IL-13 was used at 40ng/ml and mOSM at 25ng/L (R&D Systems).

Immunodetection

For immunohistochemistry, mammary gland sections were deparaffinised and boiled in 10 mM Tri-sodium citrate buffer, pH 6.0, for 10 minutes. Sections were blocked in 10% normal goat serum (Dako) for 1 hour at room temperature, incubated with primary antibody overnight at 4°C and detected using AlexaFluor-conjugated secondary antibodies (Invitrogen). For immunocytochemistry, cultured cells were fixed in 4% paraformaldehyde, permeablised with 0.25% TritonX-100, blocked with 5% milk in PBS for 1 hour and immunostained as above. Immunoblotting was as previously described (23). Antibodies against pStat3, Stat3, pStat5, Stat-5 (Cell signaling) pStat6, β-actin (Abcam), PML (Alexis), E-cadherin (BD Bioscience), Stat-6, ERα (Sigma), p63, SMA (Neomarkers) CK18 (PROGEN Biotechnik) and Transferrin receptor (Zymed Labs) were used to probe for specific antigens.

Flow cytometry and colony assays

Mammary glands from 8-14 week-old virgin mice were dissected and mammary epithelial cell suspensions prepared as previously described (31). Primary antibodies used: biotinylated anti-CD45, anti-Ter119 and anti-CD31 antibodies; anti-CD24-R-phycoerythrin (PE, clone M1/69, eBioscience), anti-CD49f-AlexaFluor-647 (Clone GoH3, eBioscience), anti-CD49b- Alexa Fluor 488 (Clone HMa2, eBioscience) and Sca 1-AF647 (Clone D7, eBioscience). Secondary antibody used: Strepavidin-PE-Texas Red (Invitrogen). Dead cells were excluded by elimination of Propidium iodide (PI) positive cells. For colony-formation assays, freshly-sorted cells were first plated on irradiated feeders as described previously (31) and incubated for a further 7 days. Colonies were fixed using ice cold acetone/methanol (1:1) and visualized using Giemsa staining (Merck).

Acknowledgements

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Figure Legends

Fig. 1. PML expression during mammary gland development. (A) Murine mammary glands from virgin (V), gestation (G), lactation (L) and involution (I) time points (in days) as indicated were probed for PML protein expression by immunoblotting. PML appears as two bands corresponding to the two alternatively spliced isoforms. PML expression was compared to the epithelial cell marker E-cadherin (E-Cad). (B) Nuclear PML-NDs (red) were visualized by immunohistochemistry, with DAPI counterstain, on mammary gland sections from various stages of development. Scale bars indicate 5 μm. Note that the prominent PML staining on tissue sections indicates a superior antibody affinity to that achieved with blotting (C) RNA levels of PML isoforms 1 and 2 were measured by qrtPCR. (D) KIM2 cells were differentiated with prolactin and dexamethasone for the number of days indicated and probed for PML expression. The transferrin receptor (TR) was used as a loading control.

Fig. 2. Stat3 and Stat6 negatively regulate PML protein expression during MGD. (A) Glands from wild type (WT) and Stat3 conditional knockout (KO) mice at 24 hours involution were analyzed for PML mRNA expression by qrtPCR (upper panel). The same glands were probed for PML protein expression by immunoblotting (middle panel) and immunofluorescence (lower panel). (B) EPH4 cells were treated with oncostatin M for the indicated number of hours and probed for active Stat3 and PML expression by immunoblotting (left panel) and for PML by immunofluorescence (right panels). Glands from wild type and Stat6 null mice at 15 day gestation were probed for PML expression by immunoblotting (left panel) and immunofluorescence (right panels). (D) EPH4 cells were treated with IL-13 for indicated times and probed for active Stat6 and PML. Scale bars indicate 5 μm.
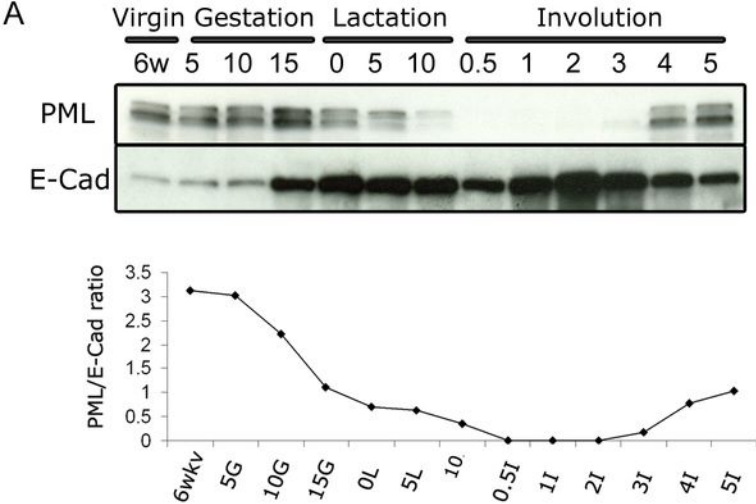
Fig 3. PML loss affects mammary gland morphogenesis. (A) Individual ducts from H&E stained sections of PML$^{-/+}$ and PML$^{-/-}$ mammary glands from virgin mice (upper panel) and whole mounts were quantified in terms of duct lumen cross-sectional area and number of branch points per duct respectively (lower panels, n=3, *** p<0.005). (B)
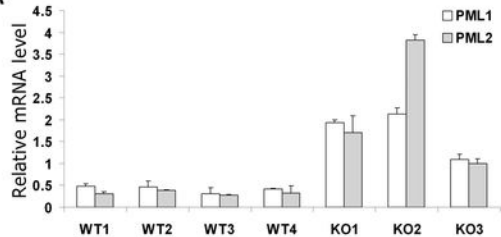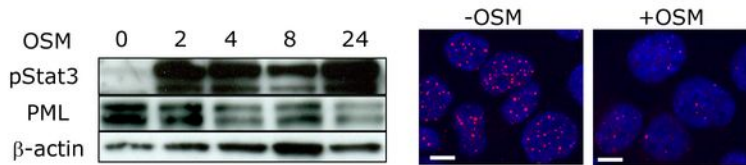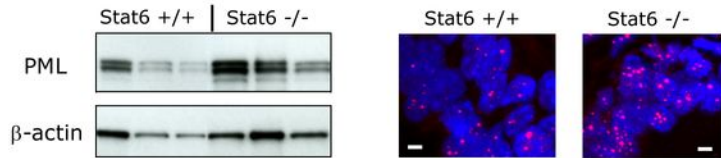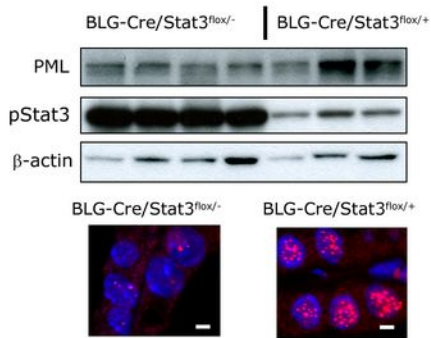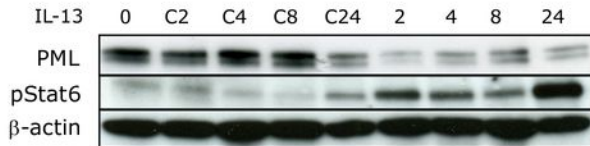
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PML$^{-/+}$ and PML$^{-/-}$ mammary glands from 15 day gestation mice were analyzed for beta-casein secretion (red) by immunohistochemical staining (upper panels – scale bars indicate 10 μm) and for Stat5 activity by immunoblotting (lower panels). A DAPI co-stain indicates nuclei.

Fig 4. PML loss influences mammary epithelial progenitor populations (A) Numbers of Sca1^{+} and Sca1^{-} luminal progenitors shown as a percentage of live luminal cells (CD24Hi) assayed by flow cytometry from 14 week old Pml$^{+/-}$ and Pml$^{-/-}$ virgin mammary glands highlighting the reduction in Sca1- and increase in Sca1+ populations in KO tissue (n=2 ***p<0.01, **p<0.02). (B) Cloning efficiency (per hundred cells) of FACS sorted Sca1^{+}, Sca1^{-} luminal progenitors and total epithelial cells from 14 week old Pml$^{+/-}$ and Pml$^{-/-}$ mice (n=2 ***p<0.01, **p<0.02). (C) Immunohistochemistry for ERα (red) and E-Cadherin (green) on mammary gland sections from 6 week old Pml$^{+/-}$ and Pml$^{-/-}$ virgin mammary glands highlighting the increase in the number of ERα^{+} cells. (D) Counts of ERα +ve cells in PML$^{+/-}$ and PML$^{-/-}$ virgin mammary glands (n=3, ***p<0.01).
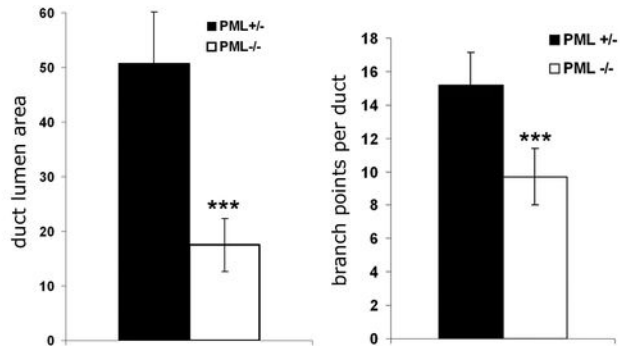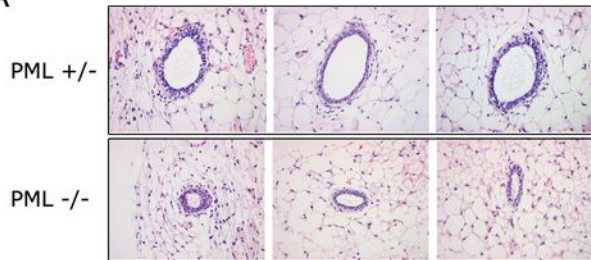
Table Legends

Table 1. Mammary epithelial differentiation markers are down-regulated in Pml$^{-/-}$ glands. Microarray analysis was carried out on mammary glands from PML$^{+/-}$ versus PML$^{-/-}$ littermates. The 9 genes found to exhibit the greatest down-regulation are displayed.
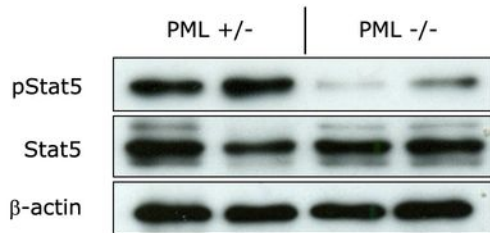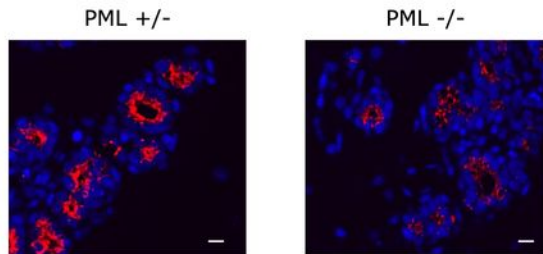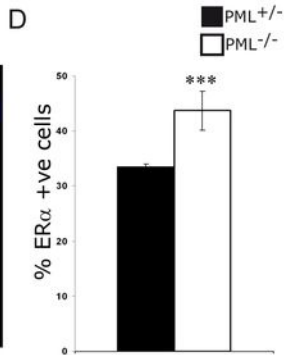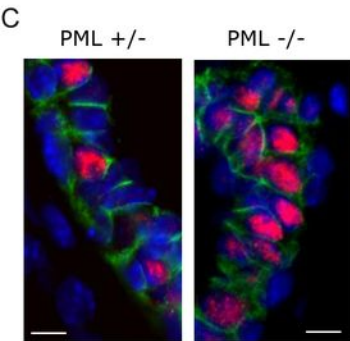
A

PML +/-

PML -/-

B

PML +/-   PML -/-

pStat5

Stat5

β-actin

A
% CD24HI cells

B
Cloning efficiency

C
PML +/-   PML -/-

D
% ERα +ve cells
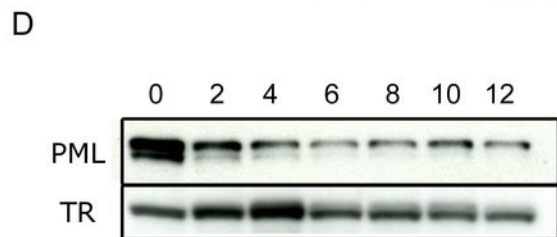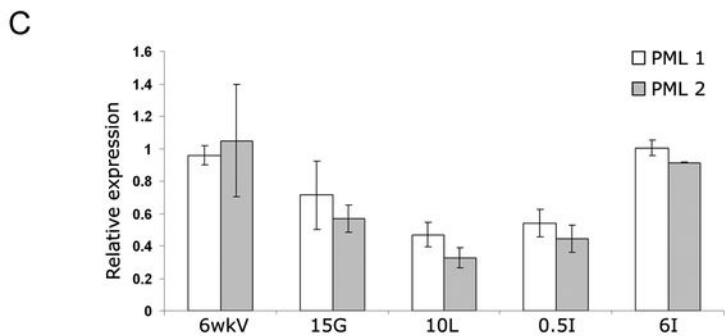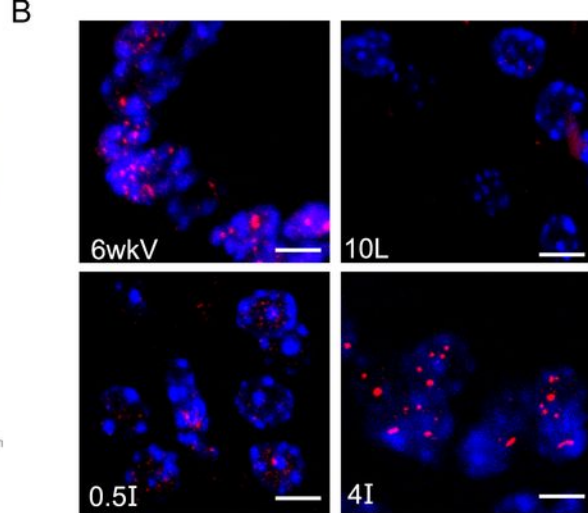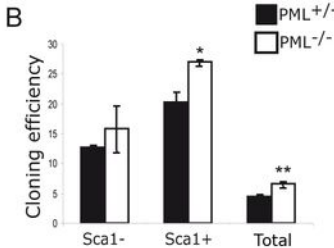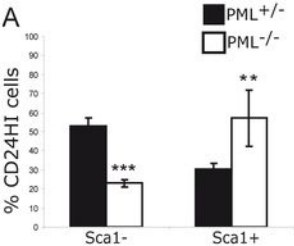
Fold Change	Gene	Gene Name
-6.80	Wap	Whey acidic protein
-4.92	Csnd	Casein delta
-4.16	1600023A02Rik	Unknown
-4.11	Krt2-8	Keratin complex 2, gene 8, CK8
-4.07	Expi	Extracellular proteinase inhibitor
-3.84	Csnk	Casein kappa
-3.24	Krt2-7	Keratin complex 2, gene 7, CK7
-2.58	Krt1-18	Keratin complex 1, gene 18, CK18
-2.53	Xist	Inactive X specific transcripts on chromosome X