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# The IL-4/IL-13/Stat6 signalling pathway promotes luminal mammary epithelial cell development

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Naïve T helper cells differentiate into Th1 and Th2 subsets, which have unique cytokine signatures, activators and transcriptional targets. The Th1/Th2 cytokine milieu is a key paradigm in lineage commitment, and IL-4 (Il4), IL-13 (Il13) and Stat6 are important mediators of Th2 development. We show here, for the first time, that this paradigm applies also to mammary epithelial cells, which undergo a switch from Th1 to Th2 cytokine production upon the induction of differentiation. Thus, the Th1 cytokines IL-12 (Il12), interferon gamma (INFγ; also known as Ifng) and Tnfα are downregulated concomitantly with the upregulation of the Th2 cytokines IL-4, IL-13 and IL-5 (Il5) as epithelial cells commit to the luminal lineage. Moreover, we show that Th2 cytokines play a crucial role in mammary gland development in vivo, because differentiation and alveolar morphogenesis are reduced in both Stat6 and IL-4/IL-13 doubly deficient mice during pregnancy. This unexpected discovery demonstrates a role for immune cell cytokines in epithelial cell fate and function, and adds an unexpected tier of complexity to the previously held paradigm that steroid and peptide hormones are the primary regulators of mammary gland development.

**KEY WORDS: Th2 cells, Cytokines, Mammary gland, Signalling, Mouse**

## INTRODUCTION

Cytokines perform crucial functions in cell fate decisions. Within the immune system, cytokines play central roles in determining the differentiation of naïve CD4$^+$ T helper (Th) cells into either of two lineages; T helper 1 (Th1) or T helper 2 (Th2) (Mosmann et al., 1986). The polarization of Th cells into either Th1 or Th2 is regulated, respectively, by IL-12 (Il12), which activates Stat4 (Jacobson et al., 1995; Thierfelder et al., 1996), and IL-4/IL-13 (Il4/Il13), which activate Stat6 (Kaplan et al., 1996). Disruption in the developmental control of Th1 and Th2 cells has been associated with diseases such as asthma, autoimmunity and cancer (Chatila, 2004; Moss et al., 2004).

Elegant experiments using various transgenic mouse models have identified factors involved in the regulation of Th2 development (reviewed in Ansel et al., 2006; Farrar et al., 2002; Murphy and Reiner, 2002). Ablation of IL-4 (Kuhn et al., 1991), Stat6 (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996) or IL-13 (McKenzie et al., 1998) expression leads to perturbed Th2 cell development and reduced type-2 immunity in mice. It is now clear that Th2 cells activate Stat6 in response to IL-4/IL-13, which, in turn, activates the transcription factor Gata3 (Zheng and Flavell, 1997). Gata3 has been shown to be essential for Th2 development because it is required for chromatin remodelling at the IL-4 locus, which facilitates transcription of the *IL-4*, *IL-13* and *IL-5* (*Il5*) genes (Takemoto et al., 1998). This can occur even in the absence of Stat6 (Ouyang et al., 2000). There are other factors that have been identified as regulators of Th2 development, such as c-Maf (Maf) and NFAT1 (also known as Nfatc2). c-Maf is a transcription factor that is expressed predominantly in Th2 cells and induces the transcription of *IL-4* (Ho et al., 1998). On the other hand, NFAT1 has been shown to be a negative regulator of Th2 development, because ablation of its expression leads to marked Th2 development (Xanthoudakis et al., 1996). The regulation of Th1/Th2 differentiation is complicated further by the involvement of the suppresser of cytokine signalling (SOCS) family of proteins. SOCS proteins are cytokine-inducible Src homology 2 (SH2)-domain-containing proteins that negatively regulate cytokine signalling (Alexander and Hilton, 2004). Socs1 and Socs3 have been implicated in negatively regulating the Th1 cytokines IFN-γ and IL-12, respectively (Egwuagu et al., 2002; Fujimoto et al., 2002; Marine et al., 1999; Seki et al., 2003). Conversely, Socs5 has been shown to be expressed in Th1 cells, where it interacts with the IL-4Rα chain and thereby attenuates IL-4 signalling, thus negatively regulating Th2 differentiation (Seki et al., 2002).

Mammary epithelial cells undergo a massive expansion in number during pregnancy. The current paradigm in mammary gland biology is that proliferation and differentiation of these epithelial cells is primarily under the control of estrogen (E), progesterone (P) and prolactin (Prl) (Hennighausen and Robinson, 2001; Rosen, 2004). Progenitor cells differentiate into either luminal or myoepithelial cells; milk is produced by the luminal cells and expelled into ducts by contraction of the myoepithelial cells. The factors that control commitment to these lineages have not been well defined.

Our previous microarray data indicated that Stat6 was abundantly expressed in mammary glands during development (Clarkson et al., 2004). Thus, we sought to address whether the Stat6 signalling pathway is involved in mammary gland development in addition to the roles of Stat5 (Liu et al., 1997) and Stat3 (Chapman et al., 1999) in different mammary developmental processes. Therefore, we characterized the cytokines, receptors and transcription factors that are involved in the Stat6 signalling pathway in both mammary gland in vivo and in mammary epithelial cells (MECs) in culture. Examination of mammary gland development in *Stat6*$^{-/-}$ and *IL-4*$^{-/-}$*/IL-13*$^{-/-}$ animals revealed a role for the Stat6 signalling pathway

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in branching morphogenesis, because development during gestation was found to be delayed. Conversely, deletion of Socs5, a negative regulator of Stat6, resulted in accelerated development. Furthermore, analysis of MECs in culture revealed a switch from Th1 to Th2 cytokine production coincident with the induction of differentiation to the luminal lineage.

## MATERIALS AND METHODS

### Mice and cell lines

*Stat6*$^{-/-}$ mice (Kaplan et al., 1996) were purchased from Jackson Laboratories and were maintained and bred in a positive pressure isolator within an SPF animal facility. Wild-type Balb/c mice were purchased from Harlan Laboratories. *IL-4*$^{-/-}$/*IL-13*$^{-/-}$ mice (McKenzie et al., 1999) and corresponding wild-type Balb/c strain-matched controls were obtained from A.N.J.M. (co-author). *IL-4*$^{-/-}$/*IL-13*$^{-/-}$ mice were maintained and bred in a positive pressure isolator. *Socs5*$^{-/-}$ mice (Brender et al., 2004) and corresponding wild-type BL/6 strain-matched controls were provided by Douglas Hilton (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). Non-obese diabetic (NOD) and NOD-severe combined immune deficiency (NOD-SCID) mice (Balb/c background) were from Anne Cooke (Department of Pathology, University of Cambridge, UK). All animals were treated according to local ethical committee and UK Home Office guidelines. Virgin female mice at 8- to 14-weeks old were mated, plug checked to confirm timing of mating and pregnancy was confirmed post-mortem to avoid pseudo-pregnancies. At least three mice of each genotype and each time point were analysed.

KIM-2 cells (Gordon et al., 2000) 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 induction, cells were grown to confluency and then differentiation media was added 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. Zero time-points were collected 24 hours post media change prior to the start of cytokine treatment. IL-4 and IL-13 (R&D) were used at the indicated concentrations (Fig. 6). EpH4 cells were grown in 1:1 DMEM:F12 supplemented with 10% FCS.

### RNA extraction and PCR primers

Mammary tissue was snap frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Tissue (100 mg) was dissolved in 1 ml of Tri-reagent (Sigma). RNA extraction was performed using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The RNA quantity and integrity was determined using a NanoDrop ND-1000 (NanoDrop Technologies). cDNA was synthesized by random hexanucleotide-primed reverse transcription from 2 μg of total RNA using the Transcriptor reverse transcription cDNA synthesis kit (Roche). Semi-quantitative detection of *Il4ra*, *Il13ra1*, *Gata3*, *Gapdh* and cyclophillin A (*CypA*, *Ppia*) was performed by PCR using Taq polymerase (Qiagen). The following primers were used (all primers are shown 5′-3′): *Il4ra* Fwd, TGGGCTGTCGATTTTGCTTTTGG, Rev, GTGCTGGGGTGGGAATCTGGTC; *Il13rα1* Fwd, GGCCATCCTGCAAAATAGTG, Rev, ACAGCGTCGGCAAGAACA; *Gata3* Fwd, TGGGTGGGGCCTCATCCTCAG, Rev, ACCGGGTCCCCATTAGCGTTCCT; *Gapdh* Fwd, CGGCAAATTCAACGGCACAGTCAA, Rev, CTTTCCAGAGGGGCCATCCACAG; and *Cyclophillin A* Fwd, CCTTGGGCCGCGTCTCCTT, Rev, CACCCTGGCACATGAATCCTG. Quantitative real-time detection of cDNA was performed using iCycler supermix (BioRad) with the addition of fluorescein (BioRad) and SYBR-green (Sigma) according to the supplier's recommendations. The real-time PCR reactions were run in an iCycler (BioRad) in triplicate. Sequences of the following primers used for real-time PCR were obtained using the PrimerBank (Wang and Seed, 2003) website (http://pga.mgh.harvard.edu/primerbank/): *Il4* Fwd, GGTCTCAACCCCCAGCTAGT, Rev, GCCGATGATCTCTCTCAAGTGAT; *Il13* Fwd, CCTGGCTCTTGCTTGCCTT, Rev, GGTCTTGTGTGATGTTGCTCA; *Il5* Fwd, CTCTGTTGACAAGCAATGAGACG, Rev, TCTTCAGTATGTCTAGCCCCTG; *Il12a* Fwd, CTGTGCCTTGGTAGCATCTATG, Rev, GCAGAGTCTCGCCATTATGATTC; *Ifng* Fwd, ATGAACGCTACACACTGCATC, Rev, CCATCCTTTTGCCAGTTCCTC; *Tnfa* Fwd, CCCTCACACTCAGATCATCTTCT, Rev, GCTACGACGTGGGCTACAG; *Gata3* Fwd, CTCGGCCATTCGTACATGGAA, Rev: GGATACCTCTGCACCGTAGC. All primers were purchased from Sigma-Aldrich. Analysis was performed using iCycler iQ Real-Time Detection System Software (BioRad). All real-time PCR products were sequenced and specificity was confirmed using BLAST.

### Whole mounts and H&E staining

For whole-mount analysis, abdominal glands (no. 4) were spread out using forceps on a glass slide and incubated in Carnoy's fixative (6 parts 100 % ethanol, 3 parts chloroform and 1 part glacial acetic acid) overnight. The slide was washed in water and placed in carmine alum stain (1 g Carmine, 2.5 g aluminum potassium sulphate and 500 ml of $dH_2O$) overnight. The slide was washed with ethanol and cleared in xylene for 1 day before documentation. For histological analysis, abdominal glands were fixed in 4% formaldehyde in PBS for 24 hours at room temperature. The glands were transferred to 70% ethanol and stored at –20°C until embedding and sectioning. All tissues were embedded in wax and sectioned at 5 μm before being stained with haematoxylin and Eosin (H&E).

### Immunoblotting

Lymph node-free abdominal mammary glands and cells were extracted with a lysis buffer containing 50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1× Cocktail protease inhibitors (Roche), 1 mM $Na_3VO_4$, and 1 mM NaF. Protein concentration was determined with the BCA colorimetric assay (Pierce) and samples were separated on criterion gels (BioRad). Immunoblotting and antibody detection using enhanced chemiluminescence (ECL, GE Healthcare) were performed as described previously (Abell et al., 2005). The following antibodies from Cell Signaling Technology were used: rabbit anti-pAkt (Akt is also known as Akt1)/PKB (Ser 473), rabbit anti-total Akt/PKB and mouse anti-pERK1/2. Other commercial antibodies used were mouse anti-ERK1/2 (Transduction Laboratories), mouse anti-pStat6 (Y641) (Abcam) rat anti-tubulin (Abcam) and goat anti-IL-4rα (R&D systems). Antibodies purchased from SCBT were: rabbit anti-Stat6 (M-20), rabbit anti-Gata3 and rabbit anti-c-Maf. Secondary HRP-conjugated antibodies were from Dako Cytomation. β-casein antibody was a kind gift from Bert Binas (Texas A & M University, Texas, TX).

### Immunohistochemistry

Paraffin-embedded mammary sections were de-paraffinized and antigen retrieval was performed using boiling 10 mM Tri-sodium citrate buffer, pH 6.0, for 10 minutes. Sections were blocked in 10% normal goat serum (normal rabbit serum in the case of IL-4rα) (Dako) for 1 hour at room temperature. Sections were either incubated with primary antibody at 1:100 for phosphorylated Stat6 (pStat6); at 1:50 for Gata3, c-Maf and IL-4Rα or with isotype control overnight at 4°C and detected using Cy3-conjugated secondary antibodies (Sigma) and bisbenzimide-Hoechst 33342 (Sigma). For intracellular KIM-2 cytokine staining, cells were grown on chamber slides (NUNC) and treated with Brefeldin-A 10 μg/ml (Sigma) for 4 hours prior to fixing in 1:1 acetone:methanol. Slides were blocked in 10% normal goat serum and stained with antibodies against IL-4 (1:50; R&D) or IL-13 (1:50; R&D).

### Microscopy and statistical analysis

Fluorescence microscopy was carried out using a Zeiss Axiovert S100TV microscope equipped with a Hamamatsu C4742-95 ORCA1 digital camera, with images visualized and manipulated using AQM 6 Advanced Kinetic Acquisition Manager software (Kinetic Imaging). The DAB IHC and H&E stains were visualized on a LEICA light microscopy. The mouse mammary gland whole mounts were visualised using the LEICA MZ75 light microscope. Quantification of images and immunoblots was performed using National Institutes of Health (NIH) ImageJ software. Statistical analysis was performed using the Sigma Stat 3.5 software package (Sysstat Software).

### Cytokine array

Medium (1 ml) from cultures of KIM-2 cells at day 0 and day 8 of differentiation was analysed using a Cytokine array I (RayBio) following the manufacturer's protocol. Chemiluminescence was detected using ECL-hyperfilm (GE Healthcare).

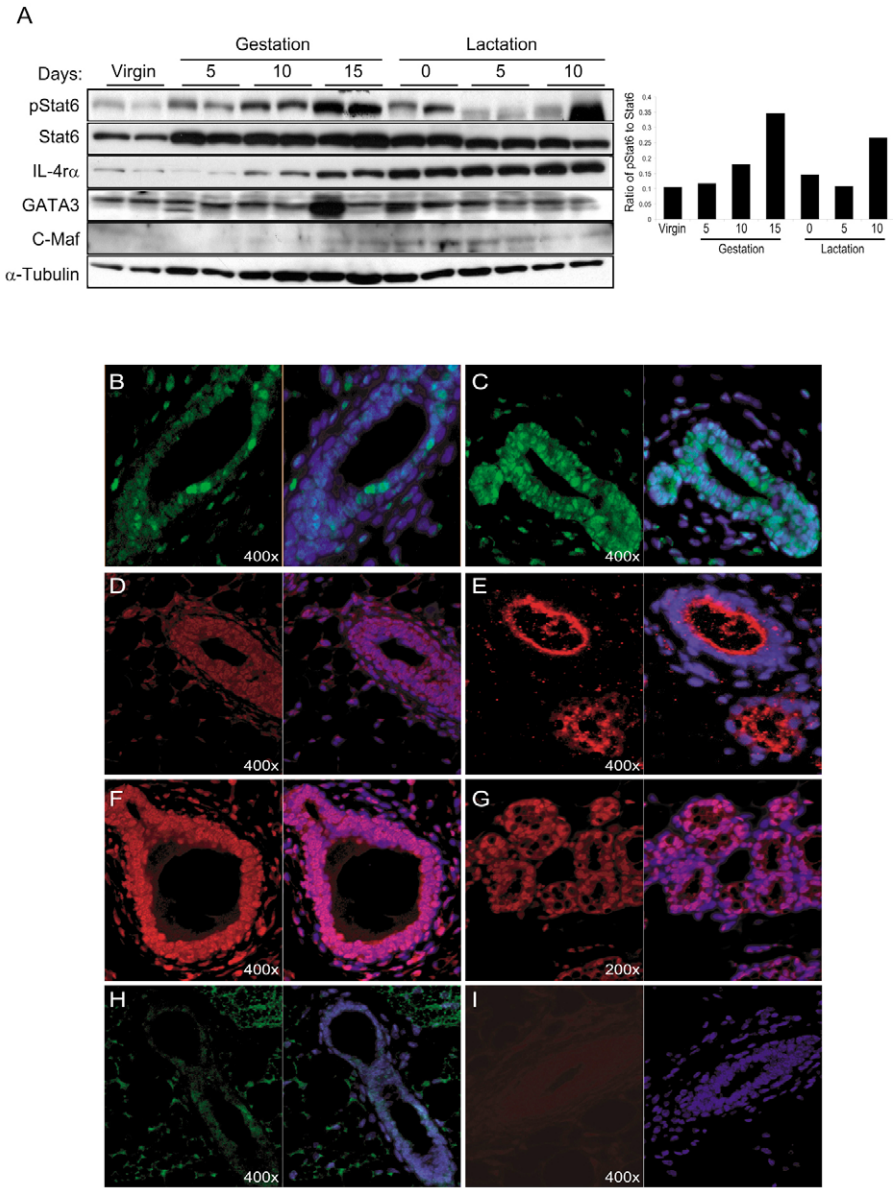



**Fig. 1. Stat6 is upregulated and activated at the onset of pregnancy.** (**A**) Protein was extracted from inguinal mammary glands from virgin mice; from mice at day 5, 10 and 15 of gestation; and from mice at day 0 (day of birth), 5 and 10 of lactation. Immunoblotting was performed for phosphorylated Stat6 (pStat6), Stat6, IL-4Rα, Gata3, c-Maf and α-tubulin. pStat6 and Stat6 immunoblots were quantified using ImageJ software and plotted on a histogram (right). (**B-I**) Immunohistochemistry (IHC, right panels; left panels, merged with DAPI staining) was performed on mammary gland sections from wild-type mice for: pStat6 in virgin mice (B) and mice at day 5 of gestation (C); IL-4Rα in virgin mice (D) and mice at day 15 of gestation (E); Gata3 in mice at day 5 of gestation (F); and c-Maf in mice at day 15 of gestation (G). (H) pStat6 staining in *Stat6*$^{-/-}$ mice at day 5 of gestation. (I) Rabbit isotype control staining on wild-type mice at day 5 of gestation.

### Microarray

cDNA from *Stat6*$^{-/-}$ mice was compared to wild-type cDNA via competitive hybridization to the arrays. A mouse exonic evidence based oligonucleotide (MEEBO) array was used (Pathology Department, Centre for Microarray Resources, Cambridge, UK). Each experiment was repeated with a dye swap for technical replicate (repeat hybridizations on separate slides using independent labelling of the same starting RNA preparations) and with three biological replicates. Total RNA from wild-type and *Stat6*$^{-/-}$ glands at day 5 of gestation was isolated using TRI reagent (Sigma) and cleaned using RNeasy columns (Qiagen), in accordance with to the manufacturer's protocols. cDNA target preparation was amplified and labelled as described by Petalidis and co-workers (Petalidis et al., 2003), except that 5 μl of $MgCl_2$ was added to the second-strand amplification reaction mix. A constant number of 14 cycles was used. For the labelling step, 2 μl of Cy3-dCTP or Cy5-dCTP was used with 22 μl of second-strand cDNA. The labelled products were purified using G50 columns, according to the manufacturer's instructions (Amersham Biosciences UK). Labelled samples were combined and hybridized for 16 hours at 50°C with 4 μl of Cot-1 DNA, 1 μl PolyA (8 μg/μl) and 1 μl yeast tRNA (4 μg/μl). Arrays were scanned using an Axon 4100A (Axon Instruments) and signal quantification was performed using Blue Fuse 3.2 (BlueGnome). Analysis was performed using FSPMA (Sykacek et al., 2005).

## RESULTS

### Stat6 signalling in MECs in vivo

The Stat6 signalling pathway has not been described previously as having a role in mammary gland development. Therefore, we performed reverse transcriptase (RT)-PCR (see Fig. S1 in the supplementary material) and immunoblots for the primary factors involved in Stat6 signalling. Phosphorylation of Stat6 was induced at day 5 of gestation and maintained until lactation (Fig. 1A). Interestingly, levels of IL-4Rα and Gata3 increased from day 10 of gestation, whereas c-Maf was increased later, from day 15 of gestation. To determine which cells express these factors, immunohistochemistry for phosphorylated Stat6 (pStat6), Gata3, c-Maf and IL-4Rα was carried out on sections of mammary tissue (Fig. 1B-I). This study revealed that pStat6 was expressed in a minority of virgin ductal epithelial cells, whereas, by day 5 of gestation, most ductal/luminal cells exhibited nuclear pStat6 (Fig. 1B,C). Interestingly, IL-4Rα expression was localised to the apical surface of luminal cells during gestation only (Fig. 1D,E), whereas the transcription factors Gata3 and c-Maf were localised in the nuclei of epithelial cells (Fig. 1F-I).

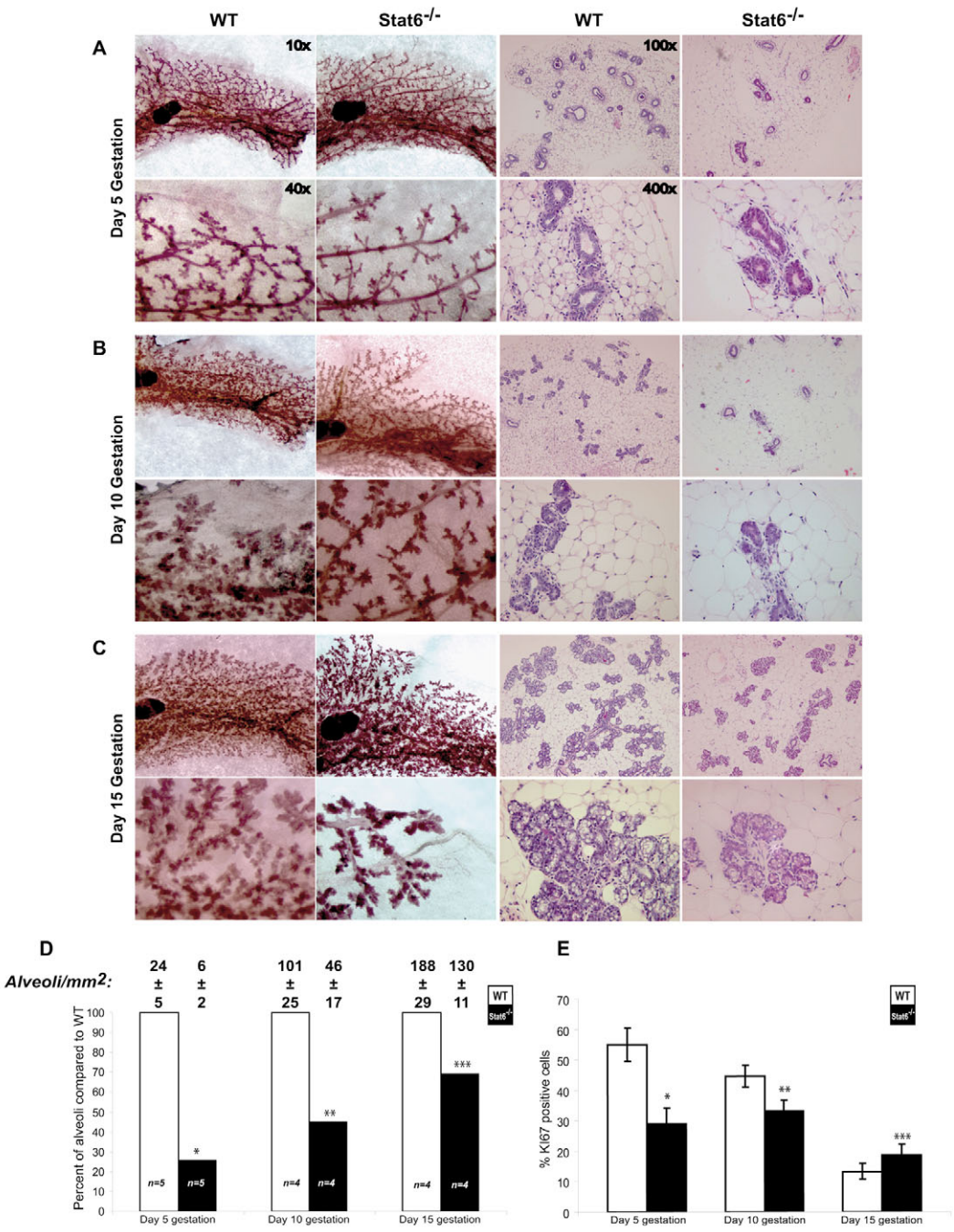



**Fig. 2. Delay in lobuloalveolar development in *Stat6*$^{-/-}$ mice.** (**A-C**) Whole mounts (left panels) and haematoxylin and Eosin (H&E)-stained sections (right panels) of mammary glands at day 5 (A), 10 (B) and 15 (C) of gestation from wild-type (WT) and *Stat6*$^{-/-}$ mice. Images shown are representative of at least three mice. (**D**) The number of alveoli was scored in H&E images from wild-type and *Stat6*$^{-/-}$ mice at 40× magnification and the area of the fat pad was measured using ImageJ software. The number of alveoli/mm$^2$ was calculated and the percentage relative to wild type was plotted. Student's *t*-test was performed and *, ** and *** indicate statistical significance with *P* values of *P*<0.001, *P*=0.011 and *P*=0.052, respectively. (**E**) Proliferation rate was measured by scoring for Ki-67-positive cells from three different fields at 400× magnification. Error bars represent s.d. of three independent mice. Student's *t*-test was performed and *, ** and *** indicate statistical significance with *P* values of *P*<0.001, *P*<0.01 and *P*<0.003, respectively.

## Stat6-deficient mammary glands exhibit delayed development

We hypothesized, based on the above data, that Stat6 would be important for normal mammary gland development. Mice deficient for Stat6 (Kaplan et al., 1996) were mated and mammary tissue harvested at various time points. Whole-mount and histological analysis of mammary glands collected from 5-week-old virgin *Stat6*$^{-/-}$ mice displayed no apparent abnormality compared to wild-type mice (see Fig. S2 in the supplementary material). However, analysis of the gestational time points showed a striking reduction in the number of side branches and alveolar buds in the absence of Stat6 at day 5 compared with strain-matched wild-type controls (Fig. 2A). At days 10 and 15 of gestation, development progressed but was delayed, and the density of the lobuloalveolar structures was diminished (Fig. 2B,C). This was clearly demonstrated in the haematoxylin and Eosin (H&E)-stained sections where the number of alveoli was approximately 70, 50 and 30% less at days 5, 10 and 15, respectively (Fig. 2D). The reduced number of epithelial cells suggests a defect in proliferation, and this was confirmed by immunostaining for Ki67, a marker of proliferation. Fig. 2E shows that the number of Ki67 positively staining cells was reduced by approximately 50% in the Stat6-deficient glands at day 5 of gestation. Interestingly, this reduction in proliferation was reversed by day 15 of gestation, suggesting a compensatory mechanism for the absence of Stat6. This 'catch-up' proliferation would account for the more subtle phenotype observed at the late gestation time points and the ability of *Stat6*$^{-/-}$ dams to nurse their pups.

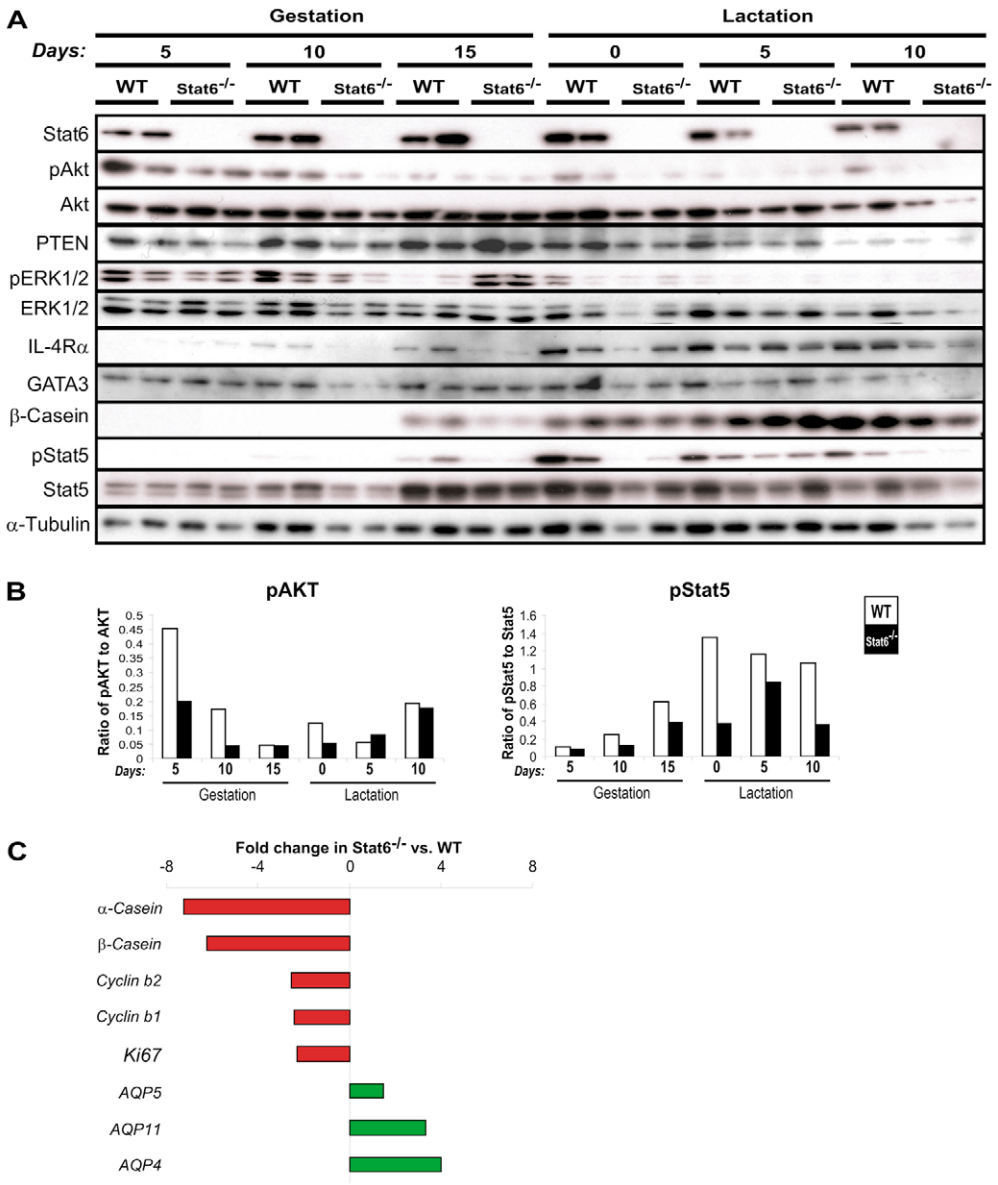



**Fig. 3. Reduction in proliferation and in the levels of differentiation markers in *Stat6*$^{-/-}$ mice.** (**A**) Immunoblots for Stat6, phosphorylated (p)Akt, Akt, pERK1/2, ERK1/2, β-casein, pStat5, Stat5, Gata3, Pten, IL-4Rα and α-tubulin were performed on wild-type (WT) and *Stat6*$^{-/-}$ tissue extracts at the indicated time points. (**B**) Immunoblots from A were quantified using ImageJ software and plotted on a histogram. (**C**) A microarray analysis of differentially regulated genes at day 5 of gestation comparing wild-type and *Stat6*$^{-/-}$ mammary glands. Genes listed displayed a statistically significant change across three biological replicates with a *P* value of <0.01.

## Multiple signalling pathways are perturbed in the absence of Stat6

A molecular analysis of the differences in proliferation/survival signalling revealed a reduction of approximately 50% in pAkt (Akt is also known as Akt1)/PKB levels in *Stat6*$^{-/-}$ glands at days 5 and 10 of gestation, with no significant change in the levels of Pten (Fig. 3A,B). This is consistent with the reduced proliferation exhibited in these glands. In addition, the Stat6 target IL-4Rα was detected at lower levels in Stat6-deficient mice at days 5, 10 and 15 of gestation, whereas this difference was less striking during lactation. However, the protein levels of Gata3, another Stat6 target, were found to be comparable between the *Stat6*$^{-/-}$ and control tissue.

The morphological delay in development in Stat6-deficient mammary glands was reflected at the molecular level by the reduced expression of the milk protein β-casein and the delayed activation of Stat5, which has been shown to be essential for lobuloalveolar development (Liu et al., 1997) and Th2 cell differentiation (Zhu et al., 2003). These changes might reflect the reduced number of fully differentiated luminal cells, because levels of the short and long forms of the prolactin receptor, PrlR, were comparable between *Stat6*$^{-/-}$ and control mice (see Fig. S3 in the supplementary material). Despite reduced levels of pStat5 and the absence of Stat6, the expression of β-casein was relatively unperturbed during lactation (Fig. 3A).

Global expression profiling of mammary tissue from day 5 of gestation *Stat6*$^{-/-}$ and wild-type glands was carried out. Changes in selected transcripts are shown in Fig. 3C. In addition to caseins α and β, which were six- to eight-fold reduced in the *Stat6*$^{-/-}$ glands, cyclins B1 and B2 and the proliferation indicator Ki67 are reduced by approximately 2-fold. Cyclin B1 and B2 are known to be expressed in dividing cells during M phase and are responsible for the induction of mitosis (Doree and Hunt, 2002). Interestingly, aquaporins 4, 5 and 11 were all expressed at higher levels in the *Stat6*$^{-/-}$ glands compared with wild type. These water transporters are known to be expressed in the ductal epithelium of virgin mice; however, during pregnancy, expression of these proteins is no longer detectable (Shillingford et al., 2003). Therefore, these microarray data provide further evidence for a delay in proliferation and differentiation in the absence of Stat6.

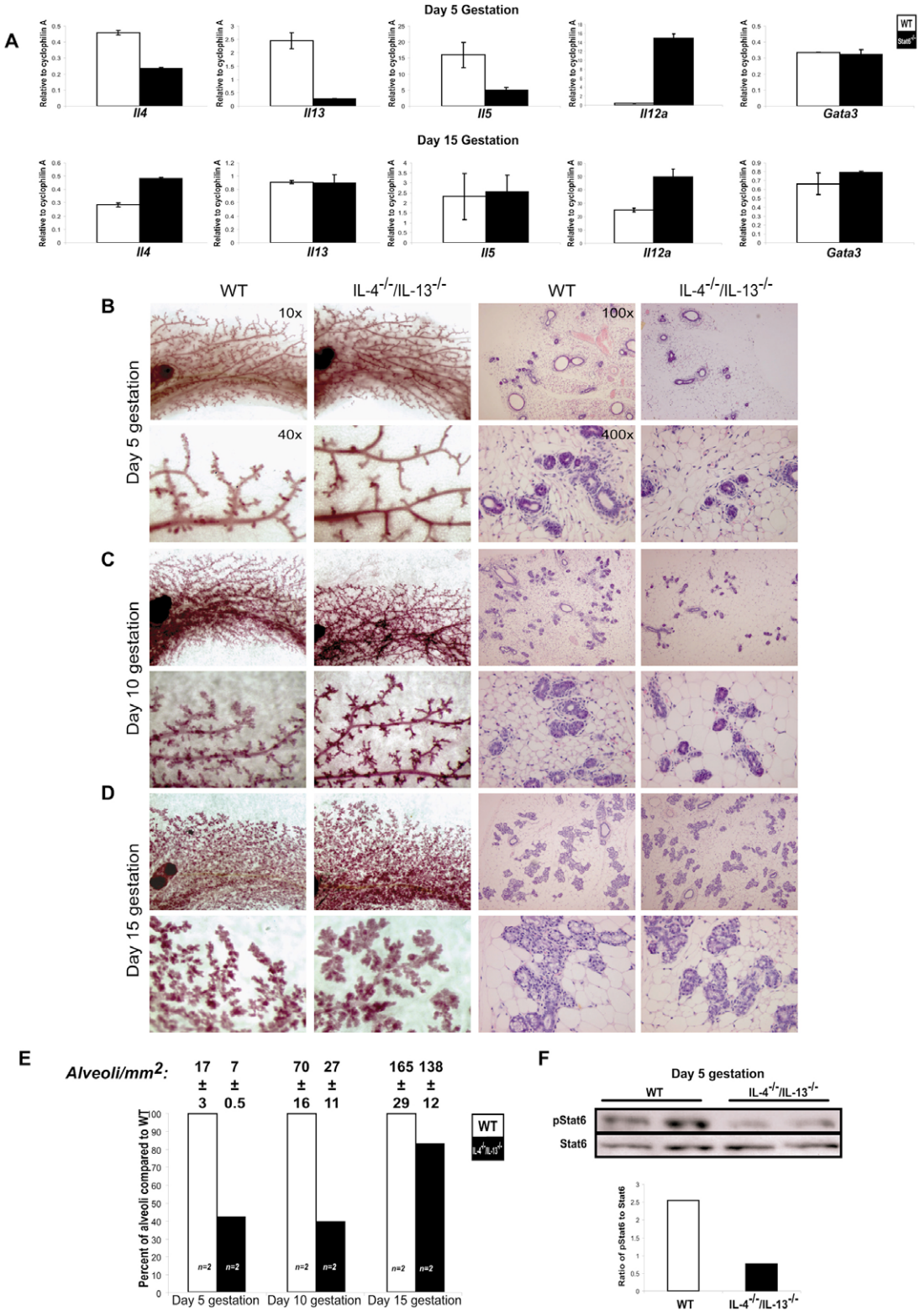



**Fig. 4. IL-4/IL-13 expression is reduced in *Stat6*$^{-/-}$ mammary glands and these cytokines are required for mammary gland development.** (**A**) Quantitative real-time-PCR was performed using specific primers for *Il4*, *Il13*, *Il5*, *Il12a* and *Gata3* on wild-type (WT) and *Stat6*$^{-/-}$ glands at day 5 and 15 of gestation. Error bar represents s.d. of three samples and is representative of at least two experiments. (**B-D**) Whole mounts (left panels) and H&E-stained sections (right panels) of inguinal mammary glands from wild-type (strain matched) and *IL-4*$^{-/-}$/*IL-13*$^{-/-}$ mice at day 5 (B), 10 (C) and 15 (D) of gestation. (**E**) The number of alveoli was scored in H&E images from wild-type and *IL-4*$^{-/-}$/*IL-13*$^{-/-}$ mice at 40× magnification and the area of the fat pad was measured using ImageJ software. The number of alveoli/mm$^2$ was calculated and the percentage relative to wild-type was plotted. (**F**) Immunoblots for phosphorylated (p)Stat6 and Stat6 were performed on wild-type and *IL-4*$^{-/-}$/*13*$^{-/-}$ mice at day 5 of gestation. The density of the bands was quantified using ImageJ software and the ratio of pStat6 to Stat6 plotted on a histogram.

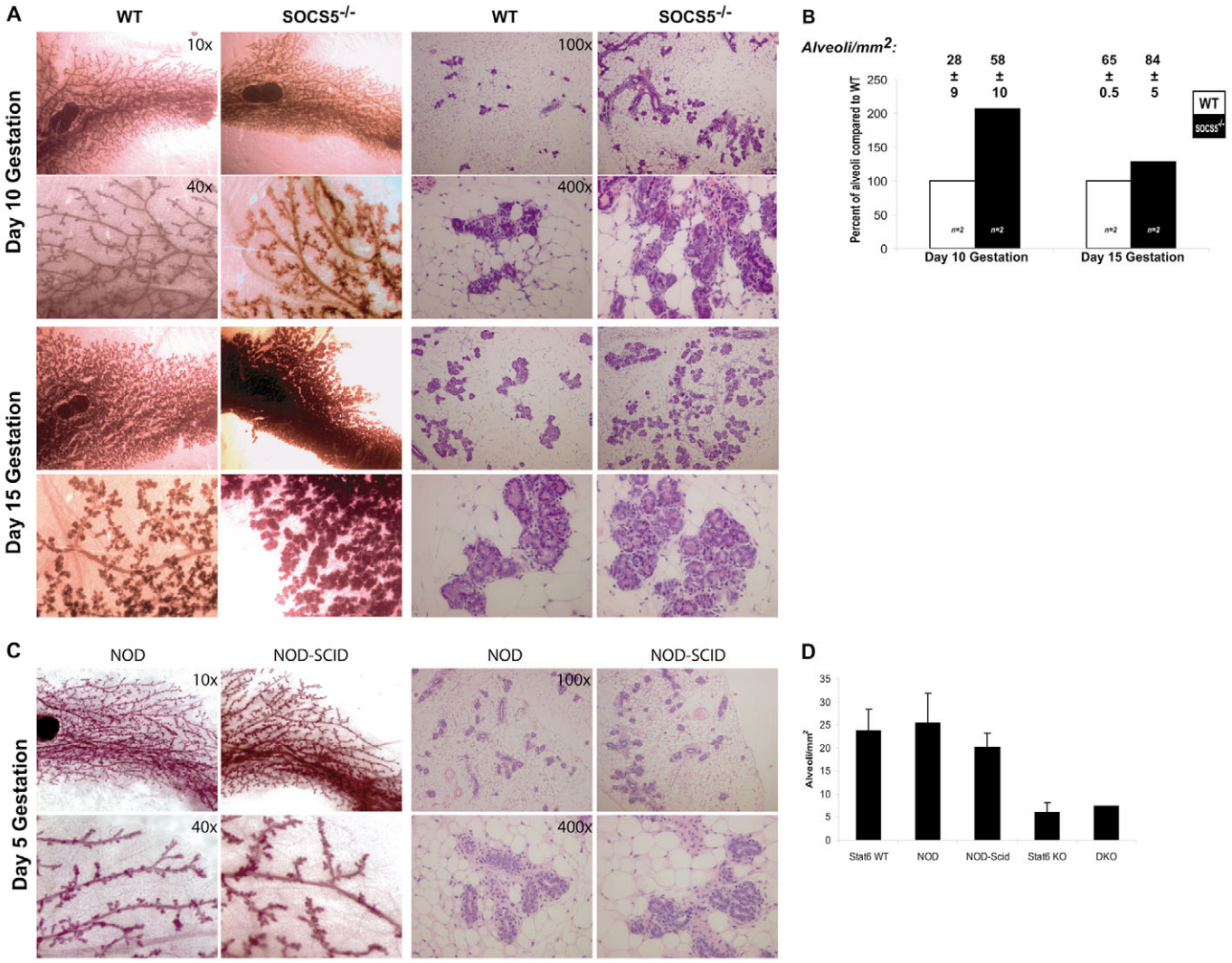
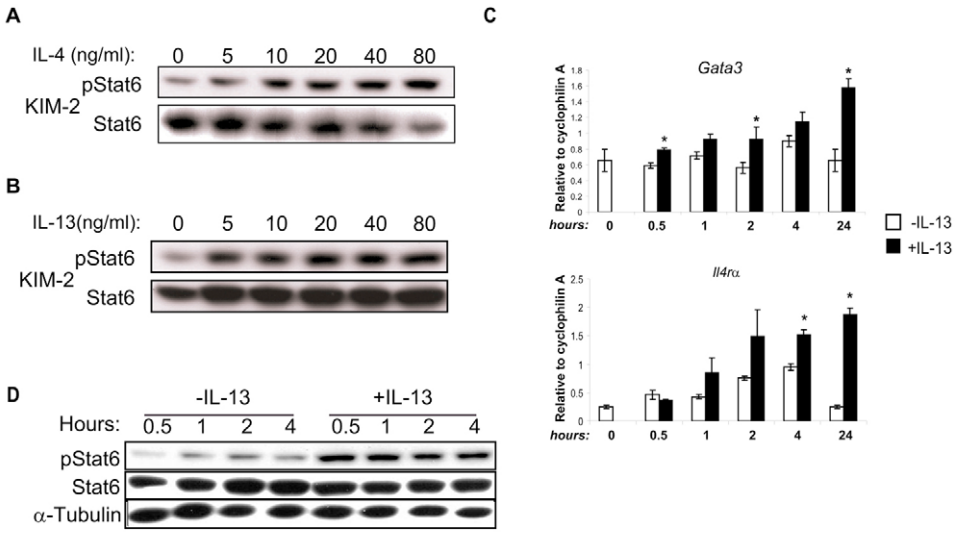



**Fig. 5. *Socs5*$^{-/-}$ mice exhibit accelerated mammary gland development.** (**A**) Whole mounts and H&E-stained sections of inguinal mammary glands from wild-type (WT) and *Socs5*$^{-/-}$ mice at day 10 and 15 of gestation. (**B**) The number of alveoli was scored in H&E images from wild-type and *Socs5*$^{-/-}$ mice at 40× magnification and the area of the fat pad was measured using ImageJ software. The number of alveoli/mm$^2$ was calculated and the percentage relative to wild-type was plotted. (**C**) Whole mounts and H&E staining of inguinal mammary glands from NOD and NOD-SCID mice at day 5 of gestation. (**D**) The number of alveoli/mm$^2$ from wild-type, NOD, NOD-SCID, *Stat6*$^{-/-}$ and *IL-4*$^{-/-}$*/IL-13*$^{-/-}$ mice at day 5 of gestation were plotted on a histogram for comparison.

## IL-4/IL-13 doubly deficient mammary glands exhibit delayed development

Stat6 is activated by a number of cytokines – particularly IL-4 and IL-13, which are also downstream targets of Stat6. However, the expression profiles of these Th2 cytokines in mammary glands are not well defined. We determined, therefore, expression levels of three Th2 cytokines – IL-4, IL-13 and IL-5 – at gestational time points by quantitative reverse transcriptase (QRT)-PCR. At day 5 of gestation, all three cytokines were reduced by between approximately 90 (*Il13*), 60 (*Il5*) and 50% (*Il4*) in the *Stat6*$^{-/-}$ samples compared with control mice (Fig. 4A). By contrast, the Th1 cytokine IL-12a was expressed at elevated levels in the *Stat6*$^{-/-}$ samples compared with control mice (Fig. 4A). By day 15, cytokine levels were no longer diminished in *Stat6*$^{-/-}$ tissue and, in fact, *Il4* was detected at higher levels compared with control mice (Fig. 4A). This reflects compensatory signalling in the absence of Stat6, which could be attributed to Gata3 because this protein is still expressed in Stat6-deficient mammary glands. IL-4 continued to be expressed throughout lactation (see Fig. S4 in the supplementary material).

The mRNA expression data from *Stat6*$^{-/-}$ mice suggest that IL-4 and IL-13 are involved in the development of the mammary gland. To address this directly, mice deficient for both cytokines (McKenzie et al., 1999) were analysed. As expected, *IL-4*$^{-/-}$*/IL-13*$^{-/-}$ mice exhibited a delay in lobuloalveolar development compared with strain-matched controls, as indicated by whole-mount and histological analysis (Fig. 4B-D). The delay was similar to that observed in Stat6$^{-/-}$ mice, with a reduced number of lobuloalveolar structures and of side branches. This is clearly demonstrated in the H&E-stained sections, where the number of alveoli was approximately 60, 60 and 16% less at days 5, 10 and 15, respectively (Fig. 4E). The phenotype observed was less severe than that of the *Stat6*$^{-/-}$ mice, which could be explained by the presence of active Stat6 in the absence of IL-4 and IL-13 (Fig. 4F).



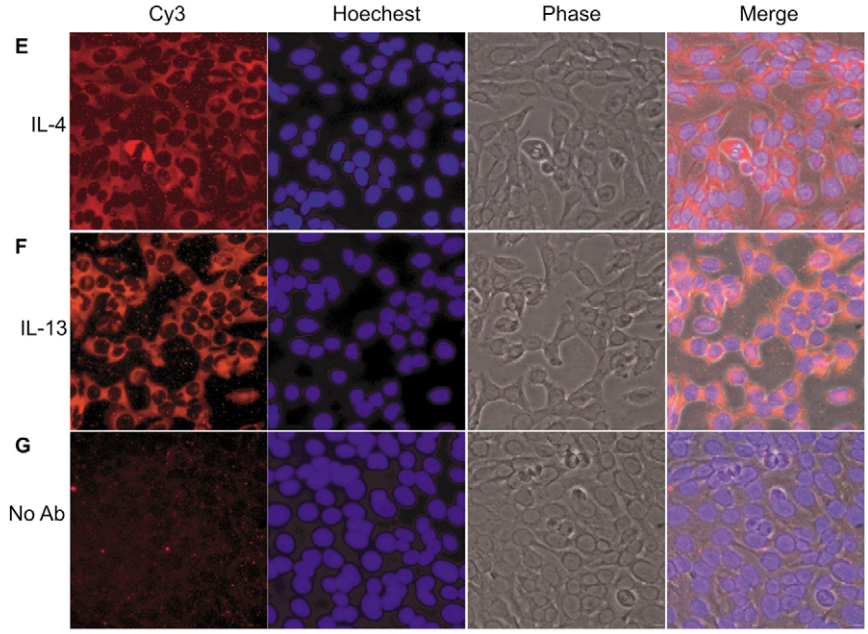




**Fig. 6. Stat6 signalling is intact in KIM-2 cells.** KIM-2 cells respond to IL-4 (**A**) and IL-13 (**B**) in a dose-dependent manner, confirmed by immunoblotting using phosphorylated (p)Stat6 and Stat6 antibodies. (**C**) KIM-2 medium was changed and then cells were harvested at various time points after treatment with or without 40 ng/ml of recombinant murine IL-13 (rmIL-13). Quantitative real-time-PCR was performed for *Il4rα1* and *Gata3*; * denotes Student's *t*-test *P* value of <0.02. (**D**) Immunoblots for the same time course were performed for pStat6, Stat6 and α-tubulin. (**E-G**) KIM-2 cells were treated with brefeldin A (10 μg/ml) for 4 hours, fixed and stained with anti-IL-4 (E) or anti-IL-13 (F) antibodies or no antibody (G).

### *Socs5*$^{-/-}$ mice exhibit accelerated mammary gland development

To confirm the notion that Stat6 signalling has a role in mammary gland development further, we investigated a model with enhanced IL-4/IL-13 signalling. Socs5 has been shown to inhibit IL-4/IL-13 signalling by binding to IL-4Rα and it is primarily expressed in Th1 cells (Seki et al., 2002). However, in *Socs5*$^{-/-}$ mice, there is no effect on B and T cell development, suggesting that Socs5 is superfluous in these cell types (Brender et al., 2004). Interestingly, we found that *Socs5*$^{-/-}$ mice have accelerated mammary gland development compared with controls (Fig. 5A), with increased numbers of lobuloalveolar structures (Fig. 5B). This suggests that enhanced IL-4/IL-13 signalling in the mammary epithelium of *Socs5*$^{-/-}$ mice results in precocious development, further supporting a role for Stat6 signalling in the regulation of mammary gland development. Taken together, these results suggest that IL-4 and IL-13 regulate Stat6 and are required for normal development of the mammary gland.

### Perturbed lymphocyte development does not affect mammary gland development

To determine whether the phenotype observed is due to the effects of IL-4/IL-13 on lymphocyte development, we investigated mammary gland development in non-obese diabetic (NOD) mice and in NOD-severe combined immune deficiency (NOD-SCID) mice. NOD mice are prone to auto-immune disease because of the high number of CD4$^{+}$ and CD8$^{+}$ T cells (Anderson and Bluestone, 2005; Prochazka et al., 1992), whereas NOD-SCID mice harbour a mutation that blocks T and B cell development (Prochazka et al., 1992). We found no defects in lobuloalveolar development in either the NOD or NOD-SCID mice at day 5 of gestation (Fig. 5C,D) and, although we did not examine later time points, other research has suggested that NOD-SCID mice have normal mammary gland development (Gupta et al., 2007). These results suggest that lymphocytes have little effect on lobuloalveolar development and that the phenotype observed in *Stat6*$^{-/-}$ and *IL-4*$^{-/-}$*/IL-13*$^{-/-}$ mice is due to epithelial cell-autonomous effects.

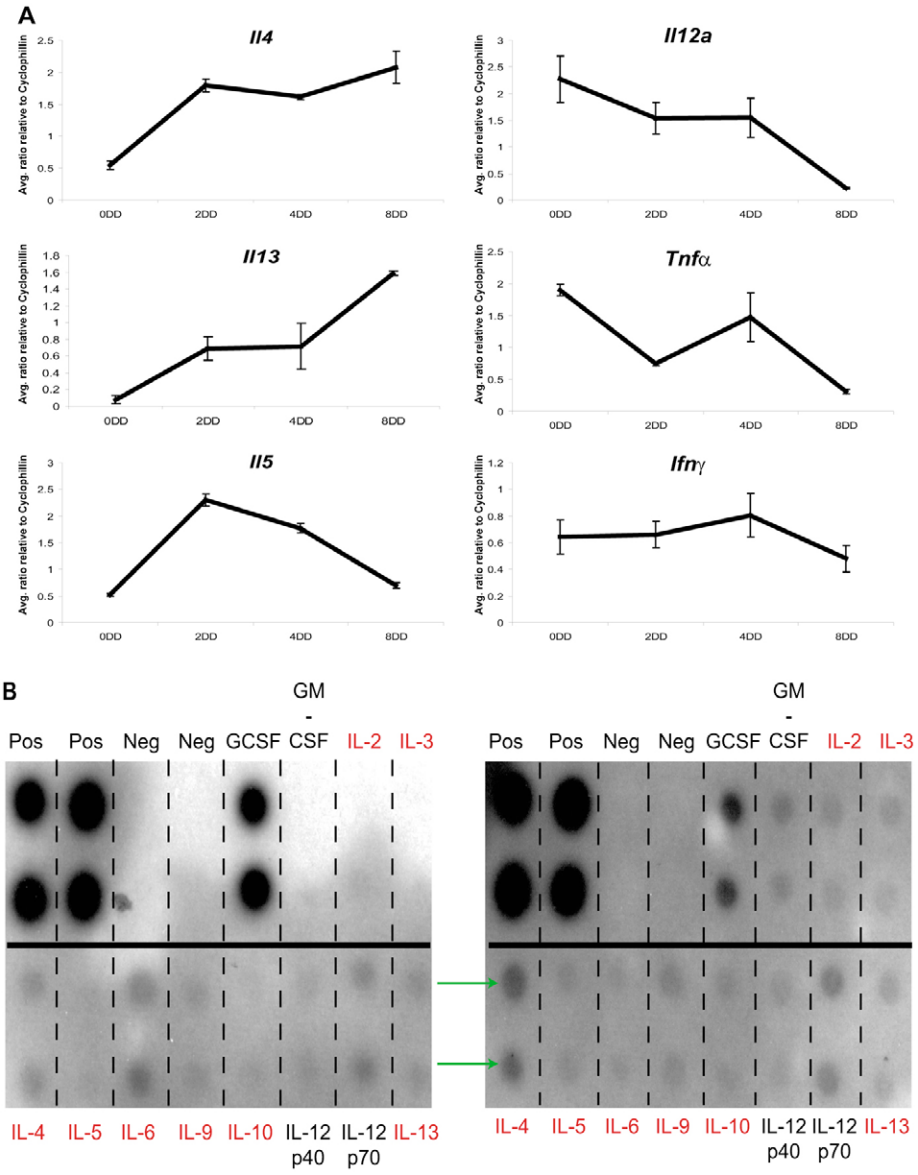



**Fig. 7. Th-2 cytokines are upregulated and secreted by differentiating KIM-2 cells.** (**A**) Quantitative real-time-PCR was performed using specific primers for the Th1 cytokines *Il12a*, *Tnfa* and *Ifng* (right) and the Th2 cytokines *Il4*, *Il13 and Il5* (left) on KIM-2 cells across a differentiation time course (DD, days of differentiation). Error bar represents s.d. of three samples and is representative of at least two experiments. (**B**) Cytokine protein array demonstrates Th2 cytokine production by differentiated KIM-2 cells at day 8 (right) compared to day 0 (left). Th2 cytokines are indicated in red. Green arrows indicate elevated levels of IL-4 secretion at day 8 of differentiation. Pos, positive control; Neg, negative control; GM-CSF, granulocyte-macrophage colony-stimulating factor; GCSF, granulocyte-colony stimulating factor.

## Stat6 signalling in MECs

To further study and confirm the Stat6 signalling observed in vivo, we studied the mouse mammary epithelial cell line KIM-2, which accurately mimics mammary gland development (Gordon et al., 2000). KIM-2 cells undergo a programme of differentiation over 10 days that is analogous to MECs in vivo (Gordon et al., 2000). Fig. S5A in the supplementary material shows the cuboidal epithelial morphology of confluent KIM-2 cells and the domes formed in response to lactogenic hormone-induced differentiation of these cells. RT-PCR analysis showed that the IL-4/IL-13 receptor components, IL-4Rα and IL-13Rα1, are expressed in undifferentiated KIM-2 cells and the expression of IL-4Rα is slightly increased after 2 days differentiation (see Fig. S5B in the supplementary material). In addition, the transcription factors Gata3 and c-Maf were detected at constant levels in KIM-2 cells throughout differentiation (see Fig. S5C in the supplementary material).

To determine whether KIM-2 cells can respond to IL-4 or IL-13 and activate Stat6, we performed a dose-response treatment with either IL-4 or IL-13 (Fig. 6A,B). Immunoblotting for pStat6 demonstrated that this signalling pathway is functional in these cells. We also examined the expression of Stat6 targets in KIM-2 cells by QRT-PCR. As shown in Fig. 6C, Gata3 and IL-4Rα are expressed at low levels in untreated cells and are induced further in response to IL-13. Gata3 expression was significantly induced after 30 minutes of treatment, whereas IL-4Rα expression was significantly higher in the treated cells after 4 hours and was elevated by 7.5-fold by 24 hours. It is noteworthy that even in the absence of recombinant IL-13, expression of Gata3 and IL-4Rα was induced, suggesting that KIM-2 cells secrete IL-13 or IL-4 into the medium. In addition, immunoblot analysis showed that Stat6 became tyrosine phosphorylated in the absence of exogenous ligand (Fig. 6D). Therefore, we treated KIM-2 cells with Brefeldin A for 2 hours to block cytokine secretion and then stained the cells using anti-IL-4 or anti-IL-13 antibodies. Importantly, we found significant levels of IL-4 and IL-13 in the cytoplasm of KIM-2 cells (Fig. 6E-G), confirming that KIM-2 cells synthesize these cytokines. It is worth noting that HC11 cells have previously been shown to produce β-casein in response to IL-4 treatment (Moriggl et al., 1997); however, no mechanism was suggested at the time.

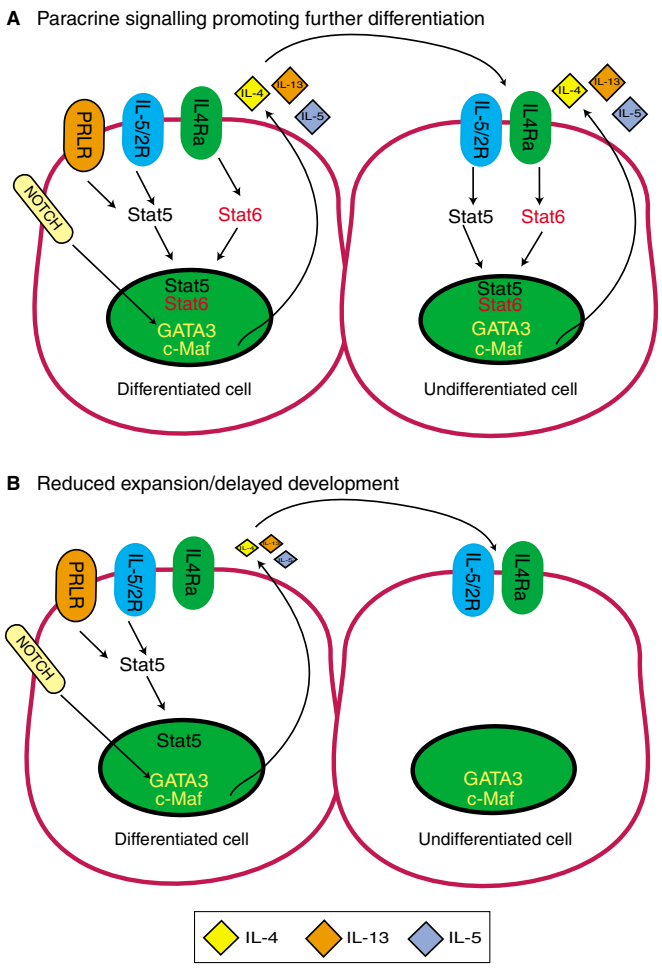



**Fig. 8. Model explaining the effect of Stat6/IL-4/IL-13 deficiency on mammary gland development.** (**A**) Th2 cytokine production is orchestrated by Stat6-mediated Gata3 gene transcription. Gata3 induces a chromatin conformational change at the IL-4/IL-13/IL-5 locus, facilitating the binding of Stat6, Stat5 and c-Maf, which produces cytokines that trigger the differentiation process in undifferentiated cells. Gata3 is also activated by other pathways, such as Notch. (**B**) In the absence of Stat6, IL-4/IL-13/IL-5 production is reduced even in the presence of Gata3. We propose that this affects paracrine signalling between neighbouring cells and thus delays development.

### Differentiation of KIM-2 cells induces a switch from Th1 to Th2 cytokine synthesis

We then investigated the expression profile of cytokines in KIM-2 cells during differentiation to the luminal (milk-producing) lineage (Gordon et al., 2000). We found a striking switch from Th1 to Th2 cytokine expression that coincided with the induction of differentiation (Fig. 7A). Thus, the Th1 cytokines IL-12a and Tnfα are significantly downregulated at day 2 of differentiation, when the milk protein β-casein is first expressed, and levels decline as differentiation progresses. By contrast, levels of INFγ (also known as Ifng – Mouse Genome Informatics), another Th1 cytokine, do not decrease until day 8, when the cells are fully differentiated (Gordon et al., 2000). Strikingly, the Th2 cytokines IL-4, IL-13 and IL-5 were immediately upregulated. It is noteworthy that the expression of IL-4 and IL-13 continued to increase during the differentiation time course, whereas IL-5 peaked at day 2 of differentiation (Fig. 7A). To confirm that these cytokines are not only synthesized by mammary epithelial cells but are also secreted, a cytokine array was used with media harvested from undifferentiated (Fig. 7B, left panel) or 8-day differentiated (Fig. 7B, right panel) KIM-2 cells. This assay showed that IL-4 is secreted at higher levels by day-8 differentiated cells than in undifferentiated cells, as are some other Th2 cytokines – IL-2, IL-3, IL-5, IL-9 and IL-10. IL-13 secretion did not appear to change. This is most probably a consequence of the rapid turnover of this cytokine. Notably, secretion of granulocyte-colony stimulating factor (GCSF) and IL-6 was diminished upon differentiation.

## DISCUSSION

Cytokines have a profound role in cell fate determination. A switch in cytokine milieu is a crucial component of T helper cell development. Cytokines that promote differentiation of the Th1 lineage suppress the Th2 lineage and vice versa. We show here, for the first time, that this paradigm also applies to differentiation of mammary epithelial cells. Expression of the Th1 cytokines IFNγ, IL-12 and Tnfα is suppressed upon lactogenic hormone-induced differentiation of MECs, whereas expression of the Th2 cytokines IL-4, IL-13 and IL-5 is induced. These data suggest that differentiation of the luminal epithelial lineage requires autocrine signalling by Th2 cytokines (Fig. 8).

This was confirmed by investigating the involvement of Th2 signalling in MECs in vivo. Mammary glands from *Stat6*$^{-/-}$ mice displayed up to a 70% reduction in side branching and delayed alveolar development during gestation, which was accompanied by reduced proliferation and reduced milk protein production. A similar phenotype was also observed in mammary glands from *IL-4*$^{-/-}$*/IL-13*$^{-/-}$ doubly deficient mice. Deletion of a negative regulator of IL-4/IL-13 would be anticipated to result in accelerated development, and the loss of Socs5 does indeed result in precocious alveolar development. This phenotype is similar to that observed in Socs1/IFNγ doubly deficient mice (Lindeman et al., 2001). Taken together, these results further support our contention that Th2-biased cytokine signalling is required for normal mammary gland development.

### Compensatory mechanisms: insights from the Th2 system

Despite the marked developmental delay in the absence of Stat6 signalling, lactation occurred normally in both *Stat6*$^{-/-}$ (see Fig. S6 in the supplementary material) and *IL-4*$^{-/-}$*/IL-13*$^{-/-}$ (data not shown) mice, suggesting that there is a compensatory mechanism that promotes functional differentiation. This scenario is similar to that observed in Th2 cells from Stat6-deficient mice, in which the Th2 cells have been shown to autoregulate Gata3 (Ouyang et al., 2000). It is also worth noting that Gata3 has been shown to be regulated by the Notch pathway in an IL-4/Stat6-independent manner (Amsen et al., 2004). Taken together, these results suggest that even low levels of Gata3 are sufficient to mediate Th2 differentiation.

We suggest that Gata3 is an important regulator of mammary gland development and that, in the absence of Stat6 signalling, Gata3 is sufficient to ultimately promote alveologenesis. While this work was under review, it was shown that Gata3 is required to maintain the differentiation of luminal epithelial cells (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006). These authors did not address the mechanism by which Gata3 is regulated in the mammary gland. Our results suggest that, similarly to Th2 cells, Stat6 signalling is required for amplification of the IL-4/IL-13 signal and for the expansion of luminal mammary epithelial cells (Fig. 8).

## Implications for cancer progression

Immune surveillance is an important factor in preventing tumour growth. It has been shown that a Th1 bias is more effective in tumour rejection (Czarneski et al., 2001; Jensen et al., 2003) and that many established tumours secrete Th2 cytokines. Significantly, estrogen receptor-positive breast tumours of a luminal subtype have been shown to express high levels of Gata3 (Usary et al., 2004). Although it is generally accepted that pregnancy is protective against breast cancer and reduces life-time risk (Medina, 2004), it is also known that a recent full-term pregnancy transiently increases the risk of developing breast cancer (Schedin, 2006). Our results provide an explanation for this apparent anomaly, because a Th2 bias during pregnancy and continued cytokine expression during lactation could hinder immune surveillance and hence promote tumour growth.

We have uncovered a novel role for Stat6 signalling in regulating differentiation of the adult mammary gland. This unexpected association between adaptive immunity and milk production suggests that, during evolution, the mammary gland hijacked an exquisitely controlled cytokine network to ensure functional differentiation. Our study reveals an additional layer of complexity to the previously held paradigm that steroid and peptide hormones are the primary regulators of mammary gland development.

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### Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/15/2739/DC1

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