A dual, non-redundant, role for LIF as a regulator of development and STAT3-mediated cell death in mammary gland

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SUMMARY

STAT3 is the key mediator of apoptosis in mammary gland. We demonstrate here that LIF is the physiological activator of STAT3, because in involuting mammary glands of *Lif*^{−/−} mice, pSTAT3 is absent and the STAT3 target, C/EBPδ, is not upregulated. Similar to *Stat3* knockouts, *Lif*^{−/−} mammary glands exhibit delayed involution, reduced apoptosis and elevated levels of p53. Significantly, *Lif*^{−/−} glands display precocious development during pregnancy, when pSTAT3 is not normally detected. We show that pERK1/2 is significantly reduced in *Lif*^{−/−} glands at this time, suggesting that at this stage LIF mediates its effects through pERK1/2. Inhibition of LIF-mediated ERK1/2 phosphorylation potentiates the pro-apoptotic effects of STAT3. LIF therefore signals alternately through ERK1/2, then STAT3, to regulate mammary growth and apoptosis.

Key words: LIF, Mammary development, STAT3, Apoptosis, ERK, Mouse

INTRODUCTION

Apoptosis plays an important role in sculpting cellular compartments during development and adult tissue homeostasis. Regulation of this cellular process is complex and requires appropriate intracellular, often transcriptional, responses to extracellular signals originating from soluble ligands (such as cytokines), extracellular matrix (integrins) and adjacent cells (cell adhesion molecules). Consequently, many of these signals activate specific transcription factors that, in turn, modulate genes encoding components of the apoptotic machinery.

We have identified a number of transcription factors that regulate apoptosis in epithelial cells of mouse mammary gland during the normal physiological process of post-lactational regression. Our work, and that of others, has highlighted the particular importance of the STAT family of transcription factors in regulating adult mammary gland development. STAT5a, which is induced by prolactin, promotes differentiation of epithelial cells and is important for lobuloalveolar development during pregnancy (Liu et al., 1997; Teglund et al., 1998). By contrast, STAT3 is pro-apoptotic and is a crucial mediator of post-lactational regression (Chapman et al., 1999). Using conditional gene targeting, we have shown that in the absence of STAT3, involution is delayed for several days, owing to a reduction in apoptosis, and this is associated with elevated levels of p53 (TRP53 – Mouse Genome Informatics) and p21, precocious activation of STAT1, and

failure to induce IGFBP5. However, unlike STAT5, the physiological inducer of STAT3 has not been identified. The aim of this study was to identify this inducer of STAT3 in mammary gland, and thereby to establish the identity of the physiological signal that initiates the involution/apoptotic switch – the crucial first step in remodelling of the mammary gland.

STAT3 is activated in response to a number of cytokines that share a common transmembrane gp130 protein receptor subunit (Heinrich et al., 1998). These include members of the interleukin (IL) 6 cytokine family, such as IL11, ciliary neurotrophic factor, oncostatin M, cardiotrophin and leukaemia inhibitory factor (LIF). STAT3 can also be activated by other factors, including prolactin, in a cell-type-specific manner.

In previous studies, we have used a cell culture model of mouse mammary gland (KIM-2) to observe signalling pathways that influence differentiation and apoptosis of mammary epithelial cells. These cells can be induced to differentiate, express milk proteins and undergo apoptosis upon withdrawal of lactogenic hormones (Gordon et al., 2000). Using these cells, we found that a number of cytokines activated STAT3, the most potent of these being LIF. LIF signalling is mediated mainly by the SHP-2/Ras/extracellular signal regulated kinase (ERK) pathway, the PI-3K/Akt pathway and JAK/STAT pathways, and can be inhibited by members of SOCS and PIAS family of proteins (Bousquet et al., 1999; Duval et al., 2000). Among the many effects of this cytokine, LIF regulates endocrine functions of the

hypothalamo-pituitary-adrenal axis and utero-placental unit (reviewed by Gadient et al., 1999), and maintains embryonic stem cell pluripotentiality (Ernst et al., 1999), haemopoiesis and neural differentiation (Kim and Melmed, 1999; Schwartz et al., 1999; Bousquet et al., 1999). Moreover, LIF can induce both differentiation and apoptosis in the M1 myeloid cell line (Minami et al., 1996; Tomida et al., 1999).

To establish whether there is a physiological role for LIF in mammary gland, we have used $Lif^{-/-}$ mice (Stewart et al., 1992). Unexpectedly, mice with an engineered null mutation for LIF develop normally and exhibit a rather mild phenotype. $Lif^{-/-}$ mice are smaller compared with control heterozygous and wild-type littermates. This mild phenotype has been attributed to partial functional redundancy between IL6 family members occurring as a result of overlapping expression patterns and the sharing of the common gp130 (Il6st – Mouse Genome Informatics) subunit. Female $Lif^{-/-}$ mice, however, are unable to support pregnancy because of defective blastocyst implantation (Stewart et al., 1992; Escary et al., 1993). We have overcome this infertility with intra-peritoneal injection of LIF post-coitus. Thus, for the first time we have been able to study complete mammary development in LIF-deficient animals.

Surprisingly, in the light of the functional redundancy between IL6 family members in other tissues, we show that LIF is the sole activator of STAT3 in mammary gland. Furthermore, we observed perturbed development that was associated with a decline in phosphorylated ERK1/2 and changes in progesterone receptor (PR) status. These findings are consistent with a dual role for LIF as the physiological activator of STAT3-mediated apoptosis during involution and ERK1/2-mediated branching morphogenesis early in mammary development.

MATERIALS AND METHODS

Generation of mice and tissues

LIF-deficient mice were generated by Dr Colin Stewart as described by Dani and colleagues (Dani et al., 1998). Implantation failure in Lif-null animals can be overcome by injection of exogenous LIF around the time of implantation (Cheng et al., 2001). $Lif^{-/-}$ animals received, on day 4 post coitum, two intraperitoneal injections of 5 µg/ml recombinant human LIF in PBS/BSA (Amrad, Richmond, Australia). Control heterozygous mice were injected using the same regime. Heterozygous LIF mice were chosen as controls, as there were no differences detected between heterozygous and wild-type mice. Genomic DNA isolated from tail tips was screened to determine the genotype by Southern blotting as previously described (Dani et al., 1998). The generation of conditional $Stat3$ knockout mice and genotyping have been described previously (Chapman et al., 1999). Briefly, mice with $Stat3$ deleted specifically in the mammary gland were generated by crossing mice with one null $Stat3$ allele and one floxed $Stat3$ allele with mice expressing Cre under the control of the β-lactoglobulin milk gene (BLG) promoter. Mice were maintained on an outbred background, control BLG-$Cre/Stat3^{flox/+}$ mice were obtained from the same colony segregating for the same combination of genotypes. Mammary glands were harvested from 4-, 5- and 7-week-old virgin $Lif^{+/-}$ and $Lif^{-/-}$ animals. Mammary tissue was also harvested from females at day 8 of pregnancy timed by the date at which vaginal plugs were first observed (designated as day 0). After injection of LIF, animals were allowed to produce litters and were maintained throughout lactation with at least six pups each. Mammary tissue was collected from $Lif^{-/-}$, $Lif^{-/+}$, $Stat3^{-/-}$ and $Stat3^{-/+}$ mice 48

hours after forced involution, initiated by removal of the pups after 10 days of lactation. All animals were sacrificed by cervical dislocation. Mammary glands were removed and either snap frozen in liquid nitrogen or fixed in formalin and embedded in paraffin wax for sectioning. Mammary whole mounts, and Haematoxylin and Eosin staining were prepared from the fourth abdominal glands and stained as previously described (Chapman et al., 1999).

Cell culture

The conditionally immortalised murine mammary epithelial cell line, KIM-2, was maintained as previously described (Gordon et al., 2000). The cells were grown to confluence in growth medium, MM (F12/DMEM supplemented with 10% FCS, 5 µg/ml insulin, 5 ng/ml EGF, and 5 µg/ml linoleic acid) and maintained at confluence for one day before treatment. For differentiation, MM was replaced 24 hour after cells had reached confluency by differentiation medium (DM) (F-12/DMEM supplemented with 10% FCS and the lactogenic hormones, 5 µg/ml insulin, 1 µg/ml prolactin, 40 ng/ml dexamethasone). Cells were cultured in DM for 12 days by which time cells expressed markers of a fully differentiated phenotype.

Detection of apoptosis

Apoptosis studies was performed using annexin V staining assessed either by flow cytometry or in situ. Undifferentiated KIM-2 cells were maintained at 100% confluence for 1 day and then incubated with either MM or MM + LIF (Peprotech, UK) with or without U0126 (10 µM, Promega, UK) for 24 hours. For annexin V flow cytometry, KIM-2 cells were harvested as previously described (Clarkson et al., 2000). For annexin V in situ, cells were stained with 1 µg/ml FITC-annexin V and after 15 minutes incubation in the dark, the plastic slides were photographed using an inverted epi-fluorescence microscope.

RT-PCR analysis

RNA was extracted from tissue using the TRIzol reagent (Promega, UK) and cDNA synthesis was performed using the Superscript cDNA synthesis kit (Gibco, URL, UK). The following forward and reverse primer pairs were used for specific amplification: Lif, 5′-CTGTGG-CTGTCATTGTTGGCGTGGTA-3′ and 5′-ATCGGCGGCGGGGT-TCGTTA-3′; $Lifr$, 5′-GGCTCTGGAACCTTGGGCAAAACTG-3′ and 5′-GCCTGCACTGCTCCAACCTCCTGTA-3′; $gp130$, 5′-TCG-GAGGAGCGGCCAGAAGAC-3′ and 5′-ATCAGCCCCCGTGCC-AAGAGC-3′; $C/EBP\delta$, 5′-ACCCGCGGCCTTCTACGA-3′ and 5′-GCGCCCTTTTCTCGGACTGT-3′; and cyclophilin, 5′-GACGCC-ACTGTCGCTTTTCG-3′ and 5′-CTTGCCATCCAGCCATTCAG-TC-3′.

The relative expression of LIF was compared between mammary tissues at different time points by real time RT-PCR using an ABI PRISM 7700 sequence detection system (Taqman) according to the manufacturer's instructions. Primers and probes were designed using the Primer Express v5.0 software (Applied Biosystems, Warrington, UK). The probe was labelled with 5′FAM and 3′TAMRA. LIF primers and probe sequences and the final concentrations they were used at were: mouse LIF forward, 5′-CCCCTGTAAATGCCACCTGT (300 nM); mouse LIF reverse, 5′-CTTCTCTGTCCCGTTGCCAT (300 nM); mouse LIF probe, FAM-5′-CATACGCCACCCATGCCACGG –3′TAMRA (200 nM).

In addition, the endogenous control 18S ribosmal RNA was assayed using primers and probe from Applied Biosystems. Probe and primer optimisation and real time PCR was performed using the manufacturer's recommended conditions. Standard curves were generated by serial dilution of a standard preparation of total RNA isolated from mouse uterus. Data are expressed in arbitrary units relative to the level of the same gene in this standard RNA. cDNA was produced from each mammary gland sample by reverse transcription using 5 µg of total RNA with 200IU Superscript RT (Invitrogen, Paisley, UK) according to the manufacturer's instructions. The expression values obtained were normalised against those from

the control ribosomal 18S to account for differing amounts of starting material.

Western blot analysis

Total protein was extracted from frozen mammary gland tissue or from KIM-2 cells in RIPA protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5), supplemented with protease and phosphatase inhibitors [10 µg/ml aprotinin, 1 µM pepstatin, 1 µM leupeptin, 1 mM phenylmethlysulfonyl fluoride (PMSF), 1 mM Na_2VO_4]. The samples were homogenised then further disrupted by passage through a 21-gauge needle (between eight and 10 times). These were subsequently incubated on ice for 30 minutes and centrifuged at 9500 g for 20 minutes at 4°C. Supernatants were transferred to a fresh tube and protein concentration was measured with the BCA colorimetric assay (Pierce). Samples (15-30 µg/lane) were run on SDS-polyacrylamide gels, blotted onto PVDF membranes and incubated with blocking solution (5% Marvel™) in TBS with 0.1% Tween 20 for 1 hour. Membranes were incubated with primary antibody diluted in blocking solution overnight at 4°C and specifically bound antibody was detected using horseradish peroxidase-conjugated secondary antibodies in conjunction with a chemiluminescent substrate (ECL; AP Biotech). Antibodies were obtained as follows: STAT3, tyrosine phosphorylated STAT3 (pSTAT3), STAT1, tyrosine phosphorylated STAT1 (pSTAT1) and phosphorylated ERK1/2 (pERK1/2) from New England Biolabs; ERK1/2 from Transduction Labs; tyrosine phosphorylated STAT5a/b (pSTAT5) from Upstate Biotechnology; p21 from Pharmingen; STAT5, Bcl-x (Bcl2l – Mouse Genome Informatics) and Bax from Santa Cruz; p53 (CM5 antibody) was a gift from Prof. David Lane (University of Dundee, UK); and β-casein was a gift from Dr Bert Binas (Berlin).

Immunohistochemistry

Immunohistochemistry for pSTAT3 was carried out with the rabbit polyclonal antibody (New England Biolabs) and the peroxide-based Envision+™ system (Dako, Ely, UK). Mammary gland sections (5 µm) were deparaffinized and subjected to antigen retrieval in a pressure cooker for 1 minute (high-pressure) and 9 minutes (low pressure) in 10 mM citrate acid buffer (pH 6.0). Endogenous peroxidase activity was inactivated by incubation in 1% hydrogen peroxide in water for 20 minutes. Sections were rinsed with TBS (25 mM Tris at pH 7.6, 130 mM NaCl) and blocked with 20% normal goat serum in TBS for 30 minutes, then incubated for 1 hour with primary antibody (diluted 1/100 in 5% normal goat serum in TBS), washed in TBS and incubated with HRP-conjugated Envision™ polymer for 30 minutes. Sections were then washed with TBS and incubated with diaminobenzidine (0.5 mg/ml in 48 mM Tris, 0.038 M HCl and 10 mM imidazole at pH 7.6 containing 0.02% hydrogen peroxide) until they turned brown. After washing with water, samples were counterstained with Haematoxylin, dehydrated and mounted. Immunohistochemistry for the progesterone receptor (PR) was carried out using a rabbit polyclonal anti-PR antibody (Dako, Ely, UK), a biotinylated goat anti-rabbit IgG second antibody and the ABC detection system (both supplied by Vector Laboratories). Briefly, 5 µm sections of mammary tissue were dewaxed, rehydrated and endogenous peroxidase blocked by incubating sections in 0.2% hydrogen peroxide in methanol. Antigen was retrieved by pressure cooking for 1 minute and 30 seconds at high pressure and the sections rinsed in phosphate-buffered saline (PBS, pH 7.0). Nonspecific binding was blocked by incubating the sections in 10% goat serum, 0.1% bovine serum albumin and avidin in PBS for 1 hour. Sections were incubated for 1 hour with the primary antibody (diluted 1:50 in 10% normal goat serum, 0.1% BSA and biotin in PBS), washed twice with PBS and incubated with biotinlyated goat ant-rabbit IgG antibody diluted 1:200 in NGS/BSA solution for 35 minutes. After washing in PBS, the ABC reagent was applied to the sections for 35 minutes, washed and incubated with diaminobenzidine for 8 minutes.

Sections were counterstained in Haematoxylin, dehydrated and mounted. PR expression was quantitated by counting at least 1000 lobulo-alveolar cells across several randomly selected high power fields and expressing the number of positively stained nuclei as a percentage of the total counted.

RESULTS

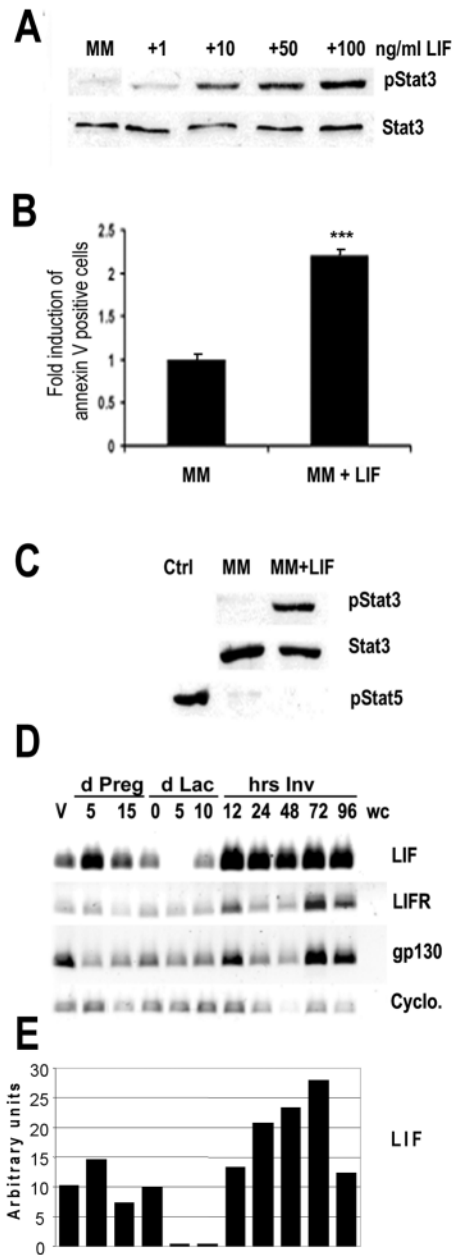### LIF activates STAT3 in a culture model of mammary gland and induces apoptosis

STAT3 is activated by tyrosine phosphorylation on Tyr-705 in response to growth factors and cytokines. We tested a variety of cytokines for their ability to induce tyrosine phosphorylation of STAT3 (pSTAT3) in KIM-2 mammary epithelial cells in culture (data not shown). STAT3 was efficiently phosphorylated by LIF in KIM-2 cells in a dose-dependent manner (Fig. 1A). Because we have shown that STAT3 is a pro-apoptotic signal for mammary epithelial cells in vivo, we determined whether LIF treatment could induce apoptosis in KIM-2 cells. Treatment of undifferentiated KIM-2 cells with LIF in growth medium (MM) for 24 hours resulted in a twofold increase in the number of apoptotic cells as assessed by annexin V staining and flow cytometry (Fig. 1B). Recently, it was reported that LIF could activate STAT5a in various cell types (Piekorz et al., 1997). In KIM-2 epithelial cells, however, LIF treatment led to phosphorylation of STAT3 but not STAT5 (Fig. 1C).

LIF, LIFR and gp130 are expressed in mouse mammary gland

The ability of LIF to activate STAT3 in mammary epithelial cells raised the possibility that LIF may activate STAT3 in the mammary gland. We therefore determined the expression pattern of LIF, LIFR and gp130 in mammary gland throughout an entire pregnancy/involution cycle (Fig. 1D). LIFR and gp130 were expressed throughout mammary development, while the expression of LIF was markedly regulated. The highest levels of LIF were seen in virgin glands, in early pregnancy and throughout involution but were noticeably reduced during lactation. The levels of LIF expression were confirmed using real-time PCR (Fig. 1E). A 33-fold upregulation of LIF expression was observed to occur between day 10 lactation and 12 hours after forced involution. This rapid and dramatic increase in LIF expression suggests that LIF may be expressed by the epithelial cells.

Lif⁻/⁻ mammary glands are characterised by delayed involution, diminished apoptosis and a lack of pSTAT3

In order to address whether LIF is required for the activation of STAT3 that normally occurs within 12 hours of involution in vivo, pregnancy was established in *Lif*⁻/⁻ mice by injecting LIF around the time of implantation. We then examined mammary glands from *Lif*⁻/⁻ mice after forced involution. Pups born to *Lif*⁻/⁻ mothers fed and grew normally indicating that lactation is not substantially perturbed in the absence of LIF. However, the absence of LIF had dramatic effects on involution (Fig. 2A). Two days after forced involution, the alveoli of *Lif*⁺/⁻ glands had started to collapse, with reappearance of adipocytes. Morphologically apoptotic epithelial cells accumulated mainly

mammary tissue from day 2 of involution. In control animals, high levels of pSTAT3 were found, whereas in the $Lif^{-/-}$ we could not detect phosphorylated STAT3 protein. Similarly, reduced total STAT3 protein levels were observed in $Lif^{-/-}$ glands compared with $Lif^{+/-}$ controls, suggesting that STAT3 is not only activated by LIF but may also be transcriptionally regulated by LIF at the onset of involution.
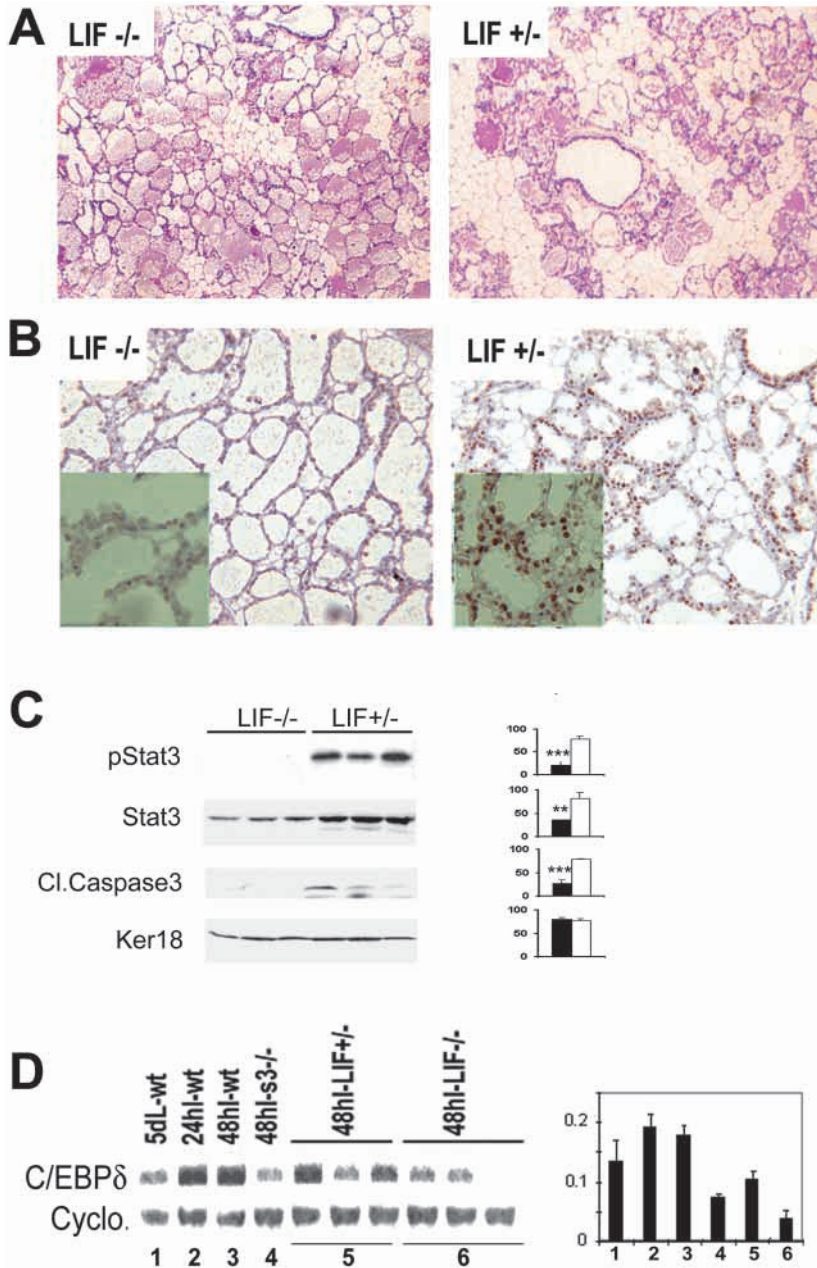
The above result was confirmed by immunohistochemistry with a specific anti-phosphotyrosine STAT3 antibody (Fig. 2B). pSTAT3 immunostaining was evident in the nuclei of epithelial cells lining the alveoli in 2 day involuting glands of $Lif^{+/-}$ animals. By contrast, pSTAT3 staining was absent in the vast majority of cells in the LIF-deficient glands, although occasional positive cells were seen.

We also examined the expression of a known downstream target of STAT3 in mammary gland, C/EBPδ (Hutt et al., 2000) (data not shown). C/EBPδ was upregulated at day 2 involution in control (Fig. 2D, lane 5), but was not induced in $Lif^{-/-}$ (lane 6) or conditional $Stat3^{-/-}$ (lane 4) mammary tissue, thus providing further evidence for the abrogation of STAT3 activity in mammary glands lacking a LIF signal.

These data combined indicate that LIF is the principal physiological activator of STAT3 during involution. This is further supported by the recent observation of Zhao and colleagues who showed that IL6, a cytokine that also signals through gp130, does not activate STAT3 during mammary involution because in IL-6 deficient mammary glands, STAT3 is phosphorylated normally (Zhao et al., 2002).

Regulation of STAT1, STAT5 and β-casein gene expression in $Lif^{-/-}$ involuting glands

LIF deficiency is not an exact phenocopy of STAT3 deficiency. We therefore investigated the molecular changes associated with the absence of LIF during involution. The activities of STAT3 and STAT5 have a reciprocal pattern of regulation during mammary gland development. We investigated the

in the alveoli, with some shed into the open lumen. Similar to $Stat3$-null mice (Chapman et al., 1999), the alveoli of $Lif^{-/-}$ mice at the same stage remained intact and extended with milk. In contrast to $Stat3^{-/-}$ mammary glands, however, a considerable number of cells were shed into the lumen, although there was no evidence of tissue remodelling. This may be a consequence of reduced collagen deposition around ductal and alveolar structures in the absence of Lif (data not shown). Western blots for cleaved caspase 3, a late event in the apoptotic cascade, confirmed that apoptosis was indeed reduced in the absence of LIF (Fig. 2C).

If LIF is the principal activator of STAT3 in vivo, the absence of LIF should result in a failure to activate STAT3. We therefore analysed mammary samples from $Lif^{-/-}$ and $Lif^{+/-}$ mice for phosphorylated STAT3. Fig. 2C shows a representative western blot for levels of activated STAT3 in

Fig. 2. Morphological and molecular changes associated with LIF deficiency in involuting mammary glands. (A) Delayed involution in *Lif*⁻/⁻ mammary glands compared with controls. Haematoxylin and Eosin staining of mammary gland sections from *Lif*⁻/⁻ and *Lif*⁺/⁻ mice at day 2 involution. (B) Absence of pSTAT3 in involuting glands from LIF-deficient animals. Immunohistochemistry on sections of mammary glands from animals at 2 day involution using antibodies against pSTAT3. (C) LIF deficiency at 2 day involution is associated with changes in pSTAT3 and cleaved caspase 3 levels. Levels of pSTAT3, STAT3, cleaved caspase3 and the epithelial marker protein cytokeratin 18 (Ker18) were assessed by western blot analysis. Graphs indicate densitometry analysis for three independent mice, mean±s.e.m.; *Lif*⁻/⁻ ,black bars; *Lif*⁺/⁻ ,white bars. (D) Regulation of C/EBPδ transcription in glands from LIF-deficient mice at day 2 of involution. RT-PCR analysis of C/EBPδ expression in glands from wild-type mice (lane 1-3), *Stat3*⁻/⁻ mice (lane 4), *Lif*⁺/⁻ control mice (lane 5) and *Lif*⁻/⁻ mice (lane 6). The glands were harvested at 5 day lactation (lane 1), 24 hours involution (lane 2) and 48 hours involution (lane 4-6). Cyclophilin (Cyclo.) was used as an internal control. Graph indicates densitometry analysis normalised to cyclophilin, mean±s.e.m., *n*=3.

levels of STAT5a in *Lif*⁻/⁻ compared with *Lif*⁺/⁻ glands by western blot analysis. Consistent with *Stat3*-null mammary tissue, no differences were observed in the levels of STAT5 protein between *Lif*⁻/⁻ and control animals, whereas levels of pSTAT5 were higher in the *Lif*⁻/⁻ compared with *Lif*⁺/⁻ tissue (Fig. 3A). This is reflected in the higher levels of β-casein seen in the *Lif*⁻/⁻ glands and provides further evidence of a delay in involution in the absence of LIF.

STAT1 is normally activated during the late stages of involution and we observed precocious activation of STAT1 in the mammary glands of *Stat3*⁻/⁻ mice (Chapman et al., 1999). However, no phosphorylation of STAT1 was observed at day 2 of involution in either *Lif*⁻/⁻ or *Lif*⁺/⁻ glands and there were no changes in the levels of total STAT1 protein (Fig. 3A). This may be explained by a hierarchy of STAT binding to the gp130

receptor component. In the absence of STAT3 protein (as in the conditional *Stat3*⁻/⁻ glands), STAT1 binds and becomes phosphorylated. In the absence of LIF, both STAT3 and STAT1 proteins are present but neither engages with the unstimulated gp130 receptor.

Molecular analysis of apoptosis-related proteins in involuting mammary glands in the absence of LIF

Bcl2 family members have been shown to be regulated at the onset of involution (Heermeier et al., 1996; Li et al., 1997) and Bcl-x$_L$ is regulated by STAT3 in some tissues. However, it is not clear if this anti-apoptotic member of the Bcl2 family of proteins is directly regulated by STAT3 in the involuting mammary gland because only subtle changes were detected in levels of Bcl-x$_L$ in Stat3-null mammary tissue (Chapman et al.,

A

B
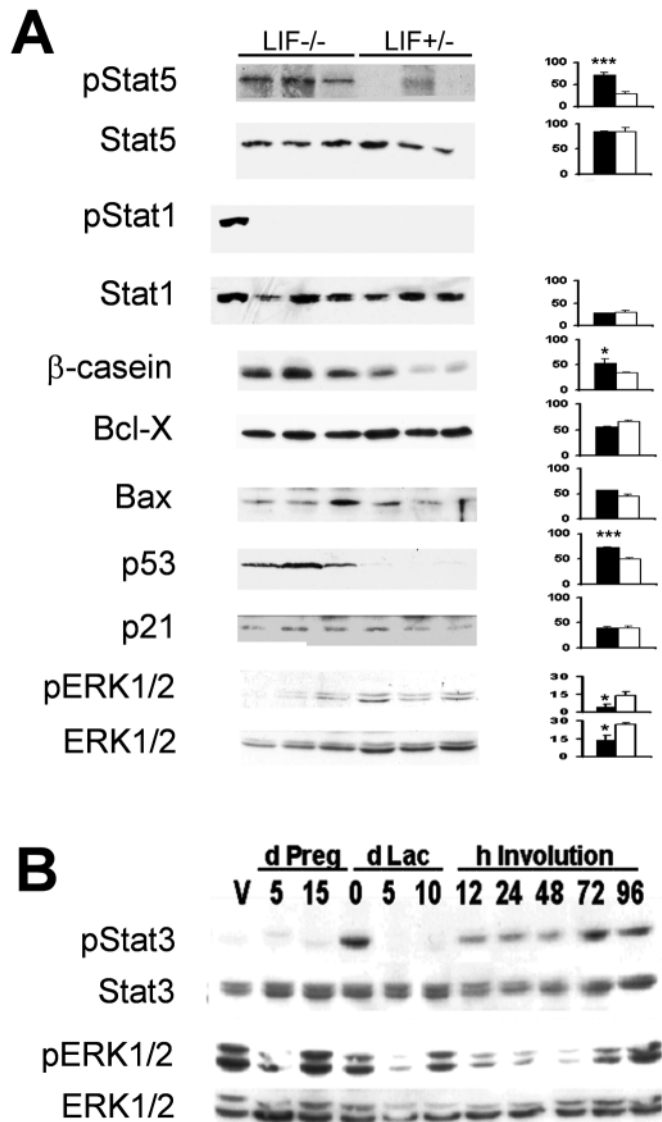
Fig. 3. Expression of apoptosis regulators in *Lif*^{-/-} and wild-type mammary gland. (A) Regulation of STAT proteins, milk protein and apoptosis related protein expression in involuting glands in absence of LIF. The levels of pSTAT5, total STAT5, pSTAT1, STAT1, β-casein, Bcl-x, Bax, p53, p21 and ERK1/2 in mammary glands from 2 day involuting mice were determined by western blot analysis. Extract from interferon gamma-treated KIM-2 cells was used as positive control for pSTAT1 and STAT1. Graphs indicate densitometry analysis for three independent mice, mean±s.e.m.. *Lif*^{-/-}, black bars; *Lif*^{+/-}, white bars. (B) Expression and activation profiles of STAT3 and ERK1/2 during mammary gland development. Western blot analysis of wild-type mammary glands harvested at indicated time points The blot was probed with antibodies specific for pSTAT3, total STAT3, pERK1/2 and total ERK1/2. d Preg, day pregnancy; d Lac, day lactation; h Involution, hours involution.

1999). No significant differences were observed in levels of Bcl-x$_L$ or Bax in Lif$^{-/-}$ compared with Lif$^{+/-}$ mice, with the exception of tissue from one animal that had elevated levels of Bax (Fig. 3A). The reason for this discrepancy is not clear.

P53 is upregulated in *Stat3*^{-/-} glands and similarly is upregulated in the *Lif*^{-/-} mammary tissue compared with

controls (Fig. 3A). P53 is a major regulator of apoptosis although its role in mammary involution is not clear because strain-dependant differences have been observed in *p53*^{-/-} mammary glands. p21 has been shown to be a target of STAT3 at the transcriptional and translational level in some cell types. It has been proposed that p21 is a survival signal in many types of cell and that active caspase 3 cleaves p21 when apoptosis is induced (Kwon et al., 2002). We showed in conditional *Stat3*^{-/-} glands that p21 is upregulated (Chapman et al., 1999). However, no changes were observed in the expression of p21 in *Lif*^{-/-} animals (Fig. 3A). This suggests that the expected upregulation of p21 in the absence of STAT3 is balanced by cleavage of p21 caused by a downregulation of survival signals in the absence of LIF receptor activation.

Interestingly, there was a significant decrease in the amount of pERK in the absence of LIF (Fig. 3A). Thus, LIF regulates pERK1/2 mediated survival signals in addition to a pSTAT3-dependent apoptotic stimulus. This regulation of pERK1/2 by LIF during involution prompted us to examine levels of pERK throughout a mammary developmental cycle (Fig. 3B). Phospho-ERK1/2 levels were highest in virgin and mid-pregnant glands. Notably, pERK levels were diminished during involution when pSTAT3 levels were highest, suggesting that at this stage LIF signals primarily through STAT3.

LIF deficiency results in aberrant ductal and alveolar morphogenesis

As pERK levels were highest early in the mammary developmental cycle, when LIF was also highly expressed, we determined whether the absence of LIF had consequences for lobuloalveolar development. Precocious alveolar development occurred in *Lif*^{-/-} glands during early pregnancy (Fig. 4A). This coincided with a significant reduction in pERK1/2 activity compared with *Lif*^{+/-} controls and an increase in pSTAT5 levels, confirming the precocious alveolar development and suggesting that LIF-induced pERK1/2 may contribute to normal development of the gland at this time. There was no significant difference in the levels of pSTAT3, which was barely detectable at day 8 pregnancy (Fig. 4B). Because LIF is expressed at significant levels in the mammary glands of virgin animals, it was interesting to determine if the absence of LIF had consequences for postnatal mammary development. In *Lif*^{-/-} mammary glands as early as 4 weeks of age, ductal elongation was markedly reduced and the ducts were thicker (Fig. 4C). In addition to a reduction in growth rate in the absence of LIF, the morphology of the terminal end buds was abnormal, these being more rounded and disorientated.

A twofold increase in the number of alveolar buds was also observed. Thus, the increased number of alveoli observed in glands during pregnancy may reflect the more frequent budding established during puberty or the absence of a growth suppressive effect of LIF during pregnancy.

Changes in PR status during pregnancy in LIF deficient mammary glands

The progesterone receptor (PR) is downregulated during pregnancy and it has been suggested that PR status reflects proliferation status (Clarke et al., 1997). Because we observed an increase in the number of mitotic cells in mid-pregnancy (data not shown), we determined the number of PR-positive cells in sections from *Lif*^{-/-} and *Lif*^{-/+} glands. Using
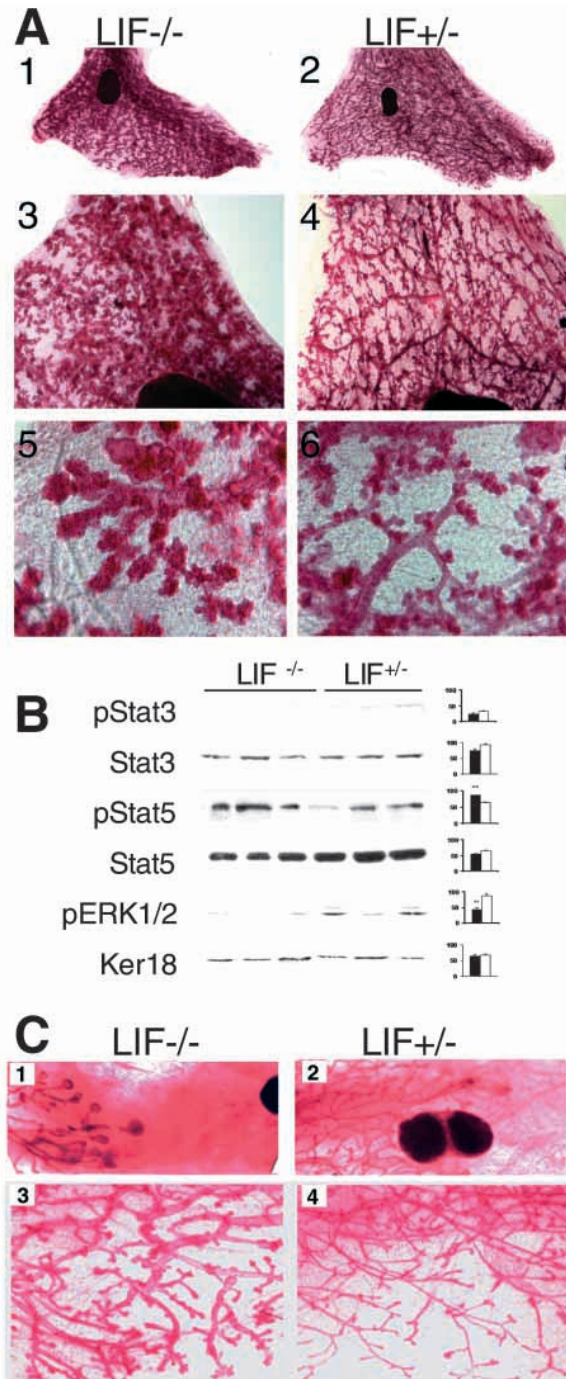
Fig. 4. Effect of LIF deficiency in early mammary gland development. (A) Whole-mount staining of mammary glands from pregnancy day 8 $Lif^{-/-}$ (1,3,5) and $Lif^{+/-}$ (2,4,6) mice at three magnifications. (B) ERK, STAT5 and STAT3 activation in early development. Glands from pregnancy day 8 $Lif^{-/-}$ and $Lif^{+/-}$ control mice were subjected to western blot analysis using specific antibodies against pSTAT3, total STAT3, pSTAT5, total STAT5, pERK1/2 and the epithelial marker protein Cytokeratin 18 (Ker18). Graphs indicates densitometry analysis for three independent mice, mean±s.e.m. $Lif^{-/-}$, black bars; $Lif^{+/-}$, white bars. (C) Whole-mount staining of mammary glands from Lif knockout (1,3) and control Lif heterozygous (2,4) virgin animals. Whole-mount staining of the glands from 4-week-old virgin mice (1,2), and 7-week-old virgin mice (3,4).

immunohistochemistry, we found that at day 8 of pregnancy, the percentage of cells expressing PR in glands from the LIF deficient mice was almost half that seen in glands from the heterozygotes (9.7±0.8 versus 16.6±1.6; P=0.018 by Student's t-test). By comparison, there was no significant difference in virgins (52.9±1.8 mean±s.e.m. versus 65.1±10.0 mean±s.e.m.). This suggests a growth restricting role for LIF during pregnancy. Other workers have previously shown that there are no ovarian defects in the $Lif^{-/-}$ mice and that progesterone levels during early pregnancy are normal (Chen et al., 2000; Song et al., 2000).

Use of a specific MEK1 inhibitor, U0126, potentiates apoptosis in undifferentiated KIM-2 cells after LIF treatment without affecting STAT3 activation

As LIF treatment of KIM-2 cells resulted in STAT3 activation (Fig. 1), we used this culture model to determine whether LIF could activate ERK also in KIM-2 cells and whether there is crosstalk between these signaling pathways in the control of apoptosis, specifically with regard to whether there is any significance in the downregulation of phospho-ERK during involution. We speculate that pERK1/2 could provide a survival signal that must be downregulated in order for STAT3 to mediate induction of apoptosis. We treated differentiated KIM-2 cells with LIF and measured levels of activated ERK1/2 and STAT3 by western blotting (Fig. 5A). Treatment with LIF resulted in a rapid activation of STAT3 (within 30 minutes) and a delayed activation of ERK. Maximal ERK activity was attained in the absence of pSTAT3, providing circumstantial evidence for crosstalk between these molecules. This was supported by in vivo evidence from $Stat3^{-/-}$ involuting mammary glands (Fig. 5B). In contrast to the decreased levels of pERK observed in $Lif^{-/-}$ glands (Fig. 3A), pERK activity was higher in the absence of STAT3 (Fig. 5B), suggesting that STAT3 may regulate the levels of pERK.

Conversely, use of the specific MEK inhibitor, U0126, to block activation of ERK1/2 resulted in abolition of pERK1/2 but did not affect STAT3 phosphorylation. This was associated with a reduction in the level of p21 (data not shown) suggesting that it is ERK that regulates p21 and not STAT3.

Moreover, there was a modest induction of apoptosis in confluent cultures of KIM-2 cells with LIF, as measured by annexin V labeling (Fig. 1B; Fig. 5C). Treatment with U0126 resulted also in a modest increase in apoptosis (Fig. 5C). However, treatment of KIM-2 cells with LIF and U0126 simultaneously, resulted in a dramatic potentiation of apoptosis, reflected by the significant increase in annexin V-positive cells. These data suggest that LIF activates signaling pathways in mammary epithelial cells which may culminate in either survival or death, depending on their relative balance and that STAT3 cannot induce apoptosis in the presence of a strong survival signal from pERK.

DISCUSSION

Despite comprehensive information now available on the effector pathways of apoptosis, comparatively little is known of the genetic mechanisms that initiate the physiological apoptosis associated with development and tissue homeostasis. There is increasing interest in mammary involution as a model
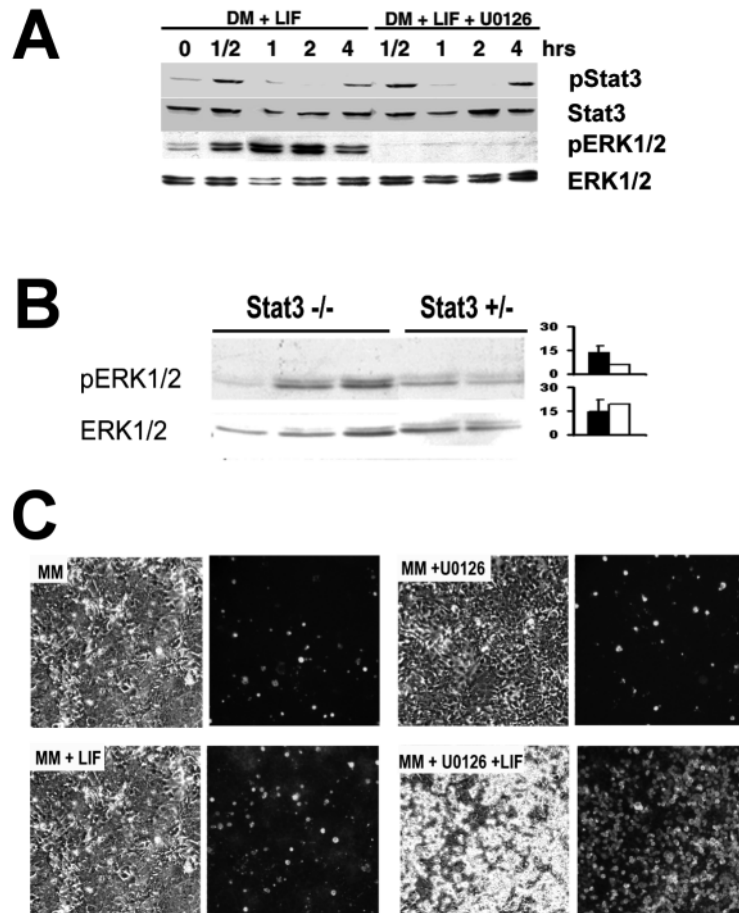
Fig. 5. ERK1/2 is activated by LIF and protects against LIF/STAT3-induced apoptosis. (A) Western blot analysis of differentiated KIM-2 cells treated with 100 ng/ml LIF for the indicated times (30 minutes, 1 hour, 2 hours and 4 hours) in differentiation medium (DM). Immunoblotting was performed using specific antibodies against pSTAT3, total STAT3, pERK1/2 and total ERK1/2. The western blot is representative of three independent experiments. (B) Increased levels of activated ERK1/2 in mammary glands from involuting STAT3-deficient mice. Levels of phospho-ERK1/2 and total ERK1/2 in 2 day involuting glands from $Stat3^{-/-}$ and $Stat3^{+/-}$ control mice was assessed by western blot analysis. Graphs indicate densitometry analysis for three independent $Stat3^{-/-}$ mice, mean±s.e.m. (black bars) and two independent $Stat3^{+/-}$ mice (white bars). (C) Phase-contrast (left) and annexin V-FITC in situ fluorescence microscopy (right) of KIM-2 cells treated with 100 ng/ml LIF in the presence or absence of 10 μM U0126 for 24 hours (bottom row) and with U0126 alone (top row). MM, time-matched control in growth medium. The results are representative of three independent experiments.

for studying these mechanisms as it is one of the most dramatic examples of a developmentally regulated apoptosis.

It has been suggested that the primary stimulus for epithelial apoptosis in the regressing mammary gland originates from locally derived factors, as involution can occur in sealed glands in the presence of normal circulating levels of hormones (Li et al., 1997). A number of potential mediators of this stimulus have been proposed, including mechanical stress of the epithelium arising from milk stasis, changing concentrations of apoptosis regulators in the milk, and immune infiltrates. We have previously reported that STAT3 has a crucial role in initiating apoptosis at the onset of mammary regression, the first description of a transcriptional regulator of epithelial apoptosis in mammary gland. In this study, we now establish that the cytokine LIF is the physiological activator of STAT3 and plays a principal role in the apoptotic process. The complete absence of pSTAT3 in $Lif^{-/-}$ glands indicates that, somewhat surprisingly, other cytokines including members of the IL6 family do not compensate for the lack of LIF signaling. The similarity between $Stat3^{-/-}$ and $Lif^{-/-}$ phenotypes in involution and the failure to induce C/EBPδ expression, a known transcriptional target of STAT3, in LIF-deficient glands further supports this conclusion.

Recently, it has been shown that LIF expression was induced by haemodynamic overload in the adult mammalian heart (Wang et al., 2001). Therefore it is possible that mechanical stretch could induce the expression of LIF, which in an autocrine manner, could then activate STAT3 and promote

apoptosis. Alternatively, LIF expression could be induced by the accumulation of a secreted factor in the lumen during milk stasis. It has been proposed that TGF-β3 may activate STAT3 during involution (Nguyen and Pollard, 2000). Overexpression of TGFβ3 in transgenic mammary glands caused precocious apoptosis and elevated levels of pSTAT3 in alveolar epithelium. However, it is unclear whether TGF-β3 directly activates STAT3 or whether it has a permissive role, through inhibition of a negative regulator of STAT3. It will be of interest to determine where TGF-β3 lies on the LIF/STAT3 axis.

Although it is not known how LIF expression is regulated during development, evidence from uterine tissue or breast cancer cell lines suggest that LIF expression could be regulated by oestrogen or progesterone (Bhatt et al., 1991; Cullinan et al., 1996; Bamberger et al., 1998). In addition, the similarities between LIF and estrogen receptor α knockout glands (Bocchinfuso et al., 2000) would suggest that either estrogen is controlling LIF expression during early development or that both those factors act simultaneously to induce mammary growth.

Expression of LIF is stage specific, being highest at the beginning (virgin and early pregnancy) and end (involution) of the developmental cycle. We demonstrate that ERK1/2 is reciprocally activated with respect to STAT3 in the mammary developmental cycle, pERK levels being highest in virgin and early pregnancy when pSTAT3 is absent, but markedly reduced at the onset of involution when STAT3 is induced. We have begun to address the molecular mechanism of this

developmentally regulated suppression of LIF-dependent STAT3 signalling in pregnancy and ERK1/2 signalling in involution. The lack of pSTAT3 in the presence of high levels of LIF in early developmental stages suggests that a specific negative regulator of STAT3 is biologically active at this time. The SOCS proteins are classic negative-feedback regulators of phosphorylated STAT proteins, of which SOCS3 is the principal target for LIF signalling (Auernhammer et al., 1998). Furthermore, SOCS3 is a direct transcriptional target of STAT3, but not STAT5 (Auernhammer et al., 1999) (data not shown). Indeed, of the four SOCS genes studied, SOCS3 exhibits an expression pattern in early development that is consistent with a role in suppressing pSTAT3 (data not shown). However, SOCS3 is also expressed at significant levels during the onset of major remodelling in the involuting gland, when pSTAT3 levels are still high. This suggests that SOCS3 is not the principal regulator of STAT3 at this stage of involution and that other mechanisms (possibly other SOCS proteins or protein tyrosine phosphatases) may regulate STAT3 at this time.

Our observations of pERK1/2 levels in $Lif^{-/-}$ and $Stat3^{-/-}$ mammary glands and in KIM-2 mammary epithelial cells treated with LIF, suggests that STAT3 promotes the downregulation of pERK1/2 in involution. We propose that the loss of ERK activity during involution is biologically significant and is necessary for STAT3 to mediate its maximal apoptotic function. Thus LIF, through STAT3, may contribute directly to the suppression of its alternate downstream signalling pathway in mammary gland. Identification of a LIF-induced molecular switch raises the question of its biological significance in mammary gland. It is interesting to speculate whether the reciprocal inhibition of STAT3 and activation of ERK1/2 early in mammary development prevents inappropriate induction of cell death as seen in our epithelial cell model.

Throughout early development we observed enlarged ducts similar to C/EBPβ-deficient mammary glands (Seagroves et al., 1998; Seagroves et al., 2000), suggesting that LIF may mediate these effects via C/EBPβ. Moreover, LIF is known to regulate C/EBPβ via ERK (Aubert et al., 1999), which is markedly reduced in LIF knockout mammary glands. Activated ERK1/2 transduces several signals including growth arrest, differentiation and survival. We observed no differences in the levels of apoptosis between LIF knockout and control animals in virgin or pregnancy mammary glands (data not shown). We also report that proliferation (and associated PR expression) is disrupted in early development in LIF-deficient mice. Although it is possible that this difference in PR expression reflects a change in cell fate arising from the increased number of alveolar cells arising during pubertal development, it is clear that LIF plays a role in growth arrest and/or differentiation, rather than apoptosis, early in development.

In conclusion, we have shown that LIF is the physiological activator of STAT3 in mammary gland and we suggest that one function of STAT3 in involution is to contribute to the downregulation of the ERK1/2 survival pathway, thus potentiating the subsequent pro-apoptotic effects of STAT3. We demonstrate a developmentally regulated bifurcation of LIF signaling via ERK and JAK/STAT pathways such that LIF signals mainly through ERK during pregnancy but through

STAT3 in involution, to control proliferation/differentiation and cell death, respectively. We speculate that this differential regulation is mediated by factors downstream of LIFR/gp130 that are expressed or regulated at different times in mammary development. SOCS3 expression early in mammary development correlates with suppression of pSTAT3 in virgin and early pregnancy (data not shown). In involution, however, LIF-dependent activation of STAT3 contributes directly to the suppression of pERK, resulting in a shift in the balance of apoptotic and survival signals. Precisely what the downstream targets of the ERK1/2 signal are, remains to be determined. However, our data clearly imply that activation of distinct pathways are required to mediate the effects of LIF at different stages of normal mammary development. The challenge now is to identify both the downstream targets of ERK1/2 in mid-pregnant mammary epithelial cells, and to further elucidate the molecular targets of STAT3 that precipitate involution.

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