

Erratum

Developmental stage determines the effects of MYC in the mammary epithelium

Collin M. Blakely, Louis Sintasath, Celina M. D’Cruz, Kristina T. Hahn, Katherine D. Dugan, George K. Belka and Lewis A. Chodosh *Development* **132**, 1147-1160.

The e-press version of the article that was published on the 2nd February contains several errors.

Both the published print and final online versions of this article are correct.

We apologise to the authors and readers for this mistake.

Developmental stage determines the effects of MYC in the mammary epithelium

Collin M. Blakely*, Louis Sintasath*, Celina M. D'Cruz, Kristina T. Hahn, Katherine D. Dugan, George K. Belka and Lewis A. Chodosh†

Departments of Cancer Biology, Cell and Developmental Biology, Medicine, and The Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6160, USA

*These authors contributed equally to this work

†Author for correspondence (e-mail: chodosh@mail.med.upenn.edu)

Accepted 9 December 2004

Development 132, 1147-1160
Published by The Company of Biologists 2005
doi:10.1242/dev.01655

Summary

Epidemiological findings suggest that the consequences of a given oncogenic stimulus vary depending upon the developmental state of the target tissue at the time of exposure. This is particularly evident in the mammary gland, where both age at exposure to a carcinogenic stimulus and the timing of a first full-term pregnancy can markedly alter the risk of developing breast cancer. Analogous to this, the biological consequences of activating oncogenes, such as MYC, can be influenced by cellular context both in terms of cell lineage and cellular environment. In light of this, we hypothesized that the consequences of aberrant MYC activation in the mammary gland might be determined by the developmental state of the gland at the time of MYC exposure. To test this hypothesis directly, we have used a doxycycline-inducible transgenic mouse model to overexpress MYC during different stages of mammary gland development. Using this model, we find that the ability of MYC to inhibit postpartum lactation is due entirely to its activation within

a specific 72-hour window during mid-pregnancy; by contrast, MYC activation either prior to or following this 72-hour window has little or no effect on postpartum lactation. Surprisingly, we find that MYC does not block postpartum lactation by inhibiting mammary epithelial differentiation, but rather by promoting differentiation and precocious lactation during pregnancy, which in turn leads to premature involution of the gland. We further show that this developmental stage-specific ability of MYC to promote mammary epithelial differentiation is tightly linked to its ability to downregulate caveolin 1 and activate Stat5 in a developmental stage-specific manner. Our findings provide unique *in vivo* molecular evidence for developmental stage-specific effects of oncogene activation, as well as the first evidence linking MYC with activation of the Jak2-Stat5 signaling pathway.

Key words: Myc, Mammary, Differentiation, Stat5, Tgfb β 3, Caveolin 1, Mouse

Introduction

A basic tenet emerging from studies in cancer biology is that normal pathways of differentiation and development are invariably disrupted during the process of carcinogenesis. This implies a fundamental relationship between these processes. The relationship between development and carcinogenesis is exemplified by the existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events such as menarche, menopause and age at first full-term pregnancy. Furthermore, epidemiological and animal studies strongly suggest that the susceptibility of the mammary gland to carcinogenesis is a function of the developmental state of the gland at the time of exposure to oncogenic stimuli. It is well established, for example, that the younger a woman is at the time of exposure to ionizing radiation, the greater her risk of eventually developing breast cancer (Boice et al., 1991; Dores et al., 2002; Hildreth et al., 1989; Land et al., 2003; Miller et al., 1989; Shore et al., 1977). In addition, numerous epidemiological studies have demonstrated that women who undergo a first full-term pregnancy early in life have a significantly reduced lifetime risk of developing breast cancer

compared with nulliparous women or with women who have their first full-term pregnancy later in life (Bain et al., 1981; Helmrich et al., 1983; Layde et al., 1989; MacMahon et al., 1970; MacMahon et al., 1982; Russo et al., 1992). These epidemiological observations predict that the consequences of a given oncogenic stimulus will vary depending on the developmental state of the mammary gland at the time of exposure. However, no molecular evidence currently exists to support this hypothesis.

Similar to the ability of the developmental state of the mammary gland to modulate its susceptibility to carcinogenesis, *in vitro* studies indicate that cellular environment can modulate oncogene action. For example, the proto-oncogene Myc has long been known to stimulate cellular proliferation. However, when constitutively expressed in growth factor-deprived cells, Myc triggers apoptosis rather than proliferation (Askew et al., 1991; Evan et al., 1992). These and other findings suggest that cellular context plays an important role in determining the consequences of Myc activation *in vitro* and are consistent with the hypothesis that the effects of aberrant oncogene

activation depend upon the specific developmental context in which activation occurs.

Beyond its *in vitro* effects, *MYC* is amplified in 5-15% of human breast cancers and is associated with aggressive tumor behavior and poor outcome (Berns et al., 1996; Chrzan et al., 2001; Deming et al., 2000; Watson et al., 1993). Consistent with a role in human breast cancer, *MYC* results in the development of invasive mammary adenocarcinomas when aberrantly activated in the mammary glands of transgenic mice (Andres et al., 1988; D'Cruz et al., 2001; Schoenenberger et al., 1988; Stewart et al., 1984). In addition, constitutive expression of *Myc* from the mouse mammary tumor virus long-terminal repeat (MMTV-LTR) has been shown to induce abnormal lobuloalveolar development in virgin mice and to inhibit postpartum lactation (Andres et al., 1988; Stewart et al., 1984). Indeed, MMTV-*myc* transgenic mice exhibit a defect in lactation that results in pup death within 24 hours of parturition. Thus, the ability of *Myc* to simultaneously disrupt normal mammary gland development and promote tumorigenesis again underscores the fundamental relationship between these two processes.

The mechanisms by which normal developmental events modulate cancer susceptibility are unknown. Understanding these mechanisms will undoubtedly require a more complete understanding of the interaction between development, reproductive history and oncogenic pathways than currently exists. To test the hypothesis that the effects of oncogene activation depend upon the developmental state of the breast at the time of exposure, we have generated a novel bitransgenic mouse model system that permits the rapidly inducible, spatially homogeneous expression of oncogenes in the mammary epithelium of bitransgenic mice treated with doxycycline (Gunther et al., 2002). This system allows regulatory molecules to be inducibly expressed in the mammary epithelium for a defined period of time, at a desired level, and during any desired developmental stage. Transgene expression is mammary specific, can be titrated over a wide range of expression levels, and is essentially undetectable in the uninduced state. Together, these properties make this system ideally suited for expressing oncogenes in a spatially and temporally restricted manner during defined stages of mammary development.

We have used this conditional transgenic model for *MYC* action to identify the specific developmental window of susceptibility that is responsible for the ability of *MYC* to inhibit postpartum lactation, as well as to determine the molecular mechanism for this phenotype. Surprisingly, we have found that *MYC* blocks postpartum lactation not by inhibiting differentiation, but rather by accelerating mammary epithelial differentiation and promoting precocious lactation during pregnancy. This in turn, results in milk stasis and premature involution of the gland mediated by the activation of Stat3 and Tgf β 3. We further show that epithelial differentiation is only accelerated when *MYC* is expressed within a discrete 72-hour period during mid-pregnancy, and that this developmental stage-specific effect is tightly linked to the ability of *MYC* to downregulate caveolin 1 expression and activate Stat5. These findings serve as a novel example of the ability of *MYC* to promote rather than inhibit differentiation, constitute the first *in vivo* example of a developmental stage-specific effect of aberrant oncogene activation, and provide the

first evidence linking *MYC* with activation of the Jak2-Stat5 signaling pathway.

Materials and methods

Transgenic mice

Generation of the MMTV-rtTA (MTB) transactivator line and the TetO-*MYC* (TOM) responder line has been previously described (D'Cruz et al., 2001; Gunther et al., 2002). The pTetO-*MYC* expression vector was generated by cloning exons 2 and 3 of human *MYC* from pSV7Humyc (Murphy et al., 1986) into pTet-Splice (Gibco BRL, Life Technologies, Rockville, MD). Bitransgenic MTB/TOM and littermate MTB control mice were administered doxycycline (2.0 mg/ml) in their drinking water to induce expression of the *MYC* transgene.

Morphological analysis

Mammary glands were fixed in 4% paraformaldehyde in 1× phosphate-buffered saline overnight and then transferred to 70% ethanol. Fixed mammary glands were embedded in paraffin and sectioned for histological staining as described (D'Cruz et al., 2001). Sections (5 μ m) were cut, applied to glass slides and stained with Hematoxylin and Eosin.

Immunohistochemistry and TUNEL analysis

Mice were injected with 1 mg BrdU per 20 g body weight 2 hours prior to tissue harvest. Mammary gland number 4 was removed and fixed overnight in 4% paraformaldehyde, transferred to 70% ethanol and embedded in paraffin wax. Sections (5 μ m) were prepared and BrdU immunohistochemistry performed as described (D'Cruz et al., 2001). Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) analysis was performed using the Apoptag Peroxidase Kit (Intergen, Purchase, New York) according to the manufacturer's instructions as previously described (D'Cruz et al., 2001). BrdU and TUNEL quantitation was performed using ImagePro software to determine the percentage of positive nuclei within a representative section. Six fields of view, and a minimum of 700 nuclei were counted per section. The standard error of the mean (s.e.m.) was calculated and a two-tailed *T*-test was performed to determine whether samples were statistically different.

Northern hybridization

Total RNA isolation and northern hybridization was performed using 3 μ g of total RNA from snap-frozen number 3 and number 5 mammary gland tissues as described (Marquis et al., 1995). Blots were hybridized with radiolabeled cDNA probes specific to exons 2 and 3 of human *MYC*, exon 1 of mouse *Myc*, epsilon-casein (*Csnd*; nucleotides 83-637), *Pip* (nucleotides 34-429), cyclin A (*Ccna2*; nucleotides 182-739), *Tgfb3* (nucleotides 2114-2568), *pai-1* (*Serpine1*; nucleotides 1467-1980), *Cdk4* (nucleotides 458-838), *Fbl* (nucleotides 39-540), *Shmt1* (nucleotides 108-546), *Cav1* (nucleotides 1414-1846), *Cis1* (*Cish*; nucleotides 188-501) and *CK18* (nucleotides 589-1287).

Western analysis

Protein lysates were prepared from mammary glands by dounce homogenization in EBC buffer as described (Gardner et al., 2000). Equivalent amounts of each extract were electrophoresed on 10% SDS-PAGE gels and transferred overnight to nitrocellulose membranes. Following visualization by Ponceau staining to verify equal protein loading and transfer, membranes were incubated with PBS blocking solution consisting of 5% nonfat dry milk and 0.05% Tween-20 (PBST-MILK). Primary antibody incubation was performed at room temperature for 1 hour with the following antibodies in PBST-MILK solution: anti-phospho-Stat5a/b (Y694/Y699) clone 8-5-2, 2.0 μ g/ml (Upstate biotechnology); anti-

Stat5, 1:250 dilution (BD Biosciences); anti- β -tubulin, 0.1 μ g/ml (InnoGenex); anti-phospho-Stat3 (Y705), clone 9E12, 0.1 μ g/ml (Upstate Biotechnology); anti-Stat3, 1:2500 dilution (Transduction Laboratories); and anti-Caveolin-1, 1:1000 dilution, clone 2297 (BD Transduction Laboratories). For milk protein westerns, membranes were blocked in a solution consisting of 3% BSA, 1 \times TBS and 0.05% Tween-20 (TBST-BSA). Polyclonal rabbit antiserum to mouse milk-specific proteins (Nordic Immunological Laboratories) was used at a 1:40,000 dilution in PBST-BSA. Following primary antibody incubations, blots were washed three times in blocking solution, and incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies at 1:10,000 dilutions (Jackson ImmunoResearch) in blocking solution for 30 minutes at room temperature. Following three washes in blocking solution and two washes in 1 \times PBS, blots were developed using the ECL Plus system according to the manufacturer's instructions (Amersham Pharmacia) followed by exposure to film (Kodak XAR-5).

Oligonucleotide microarray hybridization

Approximately 30 μ g total RNA from the mammary glands of three separate animals were used at each developmental time point. Biotinylated cRNA was generated and hybridized to Affymetrix Mu6500 GeneChips. Raw data collection and gene expression analysis was performed as described previously (Master et al., 2002).

Results

Transient MYC expression during mid-late pregnancy blocks postpartum lactation

MMTV-*myc* transgenic mice exhibit a defect in lactation that results in pup death within 24 hours of parturition. However, constitutive transgenic models of *Myc* expression cannot identify the stage of mammary gland development at which *Myc* expression results in this phenotype. In principle, the ability of *Myc* to block lactation could be due to oncogene action during puberty, when ductal morphogenesis occurs, pregnancy or lactation. To address this, we used the doxycycline-inducible transgenic mouse model, MMTV-rtTA/TetO-MYC (MTB/TOM), to conditionally overexpress MYC during discrete stages of mammary development (D'Cruz et al., 2001).

First, to determine whether the inducible MTB/TOM model that we have previously described phenocopies the lactation defect observed in MMTV-*myc* mice, we induced MYC expression in nulliparous MTB/TOM mice by administration of doxycycline beginning at 3 weeks of age. When these mice were subsequently mated at 6 weeks of age and allowed to undergo pregnancy, all pups were observed to die within 24 hours of parturition. Thus, constitutive expression of MYC in the mammary glands of MTB/TOM mice recapitulates the lactation defect observed in the constitutive MMTV-*myc* transgenic mouse model.

Deregulated expression of MYC has been demonstrated to be incompatible with terminal differentiation in multiple cell types, including myoblasts, erythroleukemia cells, adipocytes and B-lymphocytes (Brandvold et al., 2001; Coppola and Cole, 1986; Freytag, 1988; Heath et al., 2000; Miner and Wold, 1991). Consequently, we hypothesized that one mechanism by which deregulated MYC expression might inhibit postpartum lactation would be by inhibiting terminal differentiation of the mammary epithelium during this phase of development. Surprisingly, although induction of MYC during lactation did

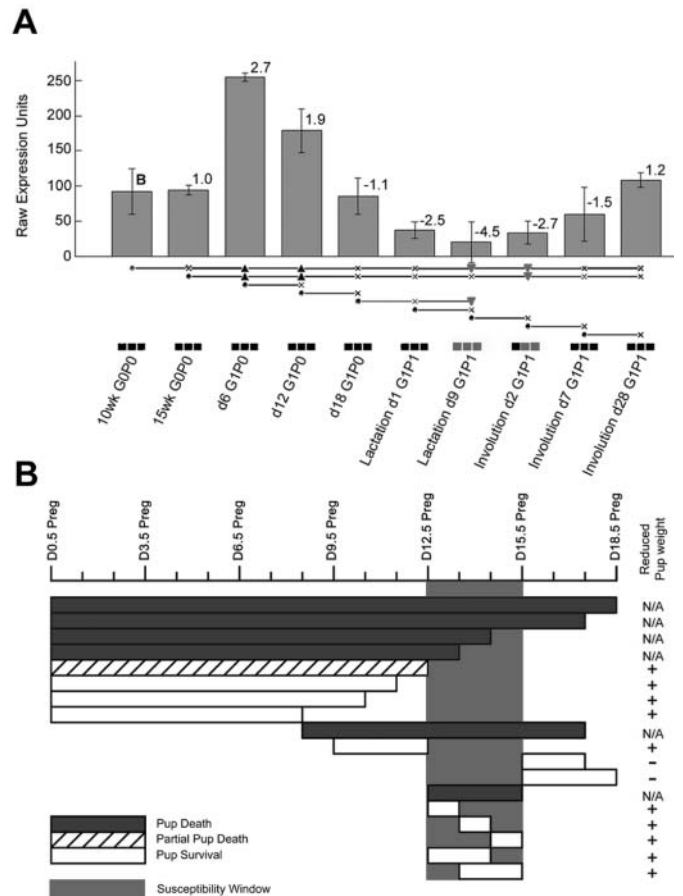


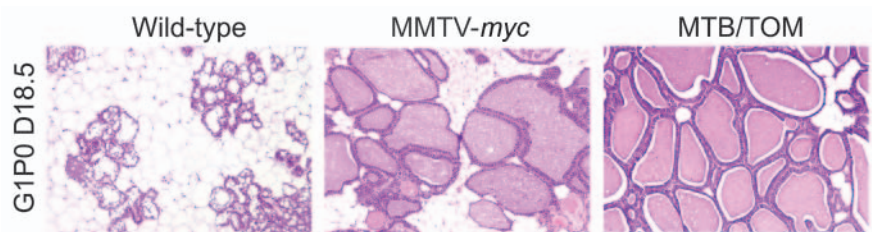
Fig. 1. Mammary epithelial expression of *Myc* during pregnancy affects pup survival postpartum. (A) Oligonucleotide microarray analysis of endogenous *Myc* expression during mouse mammary gland development. Total RNA (30 μ g) was isolated from the mammary glands of three separate FVB wild-type mice and hybridized to Affymetrix Mu6500 GeneChips as previously described (Master et al., 2002). Raw expression data are indicated on the y-axis. Black boxes indicate detectable expression, whereas gray boxes indicate samples in which *Myc* expression was not detected. Gene expression changes determined to be statistically significant by the analysis described in Master et al. (Master et al., 2005) are indicated by vertical arrowheads. \times indicates gene expression changes determined not to be statistically significant. Fold-changes in *Myc* expression, as compared with the baseline ('B') expression of 10-week-old G0P0 animals, are indicated at the top of each column. (B) Effect of *MYC* transgene expression during pregnancy on postpartum pup survival. MTB/TOM mice were administered 2.0 mg/ml doxycycline in their drinking water to induce *MYC* transgene expression during the time period of pregnancy indicated at the top. Parturition occurred after 18.5 days of gestation, and pup survival within the first 24 hours was assessed. Shaded bars indicate induction conditions for which all pups died within the first 24 hours of parturition. Non-shaded bars indicate induction conditions in which all pups survived beyond 24 hours of parturition. Hatched bars indicate induction conditions in which a fraction of the litter died within 24 hours of parturition. The effects of induction conditions on pup growth are indicated as follows: +, affected; -, not affected; N/A, not applicable because of pup death. The light-gray region from D12.5-D15.5 of pregnancy indicates the window of highest susceptibility to *MYC* induction.

eventually result in pup death, the timing of pup mortality was markedly delayed compared with litters from MMTV-*myc* mice or MTB/TOM mice induced throughout pregnancy. Similarly, deregulated MYC expression in virgin mice from 3 weeks to 7 weeks of age had no effect on pup survival in mice subsequently allowed to undergo pregnancy. These findings suggest that MYC overexpression during ductal morphogenesis or lactation is not responsible for the rapid postpartum death of pups born to MMTV-*myc* mice.

In contrast to the lack of effect of MYC overexpression during virgin development or lactation, induction of MYC expression in MTB/TOM mice from day 0.5 (D0.5) of pregnancy through day 1 postpartum (D1PP) resulted in pup death within 24 hours of parturition in a manner identical to that observed in MMTV-*myc* mice. Pups did not have milk in their stomachs, implying a maternal defect in lactation. Importantly, pups from MMTV-*myc* or MTB/TOM mothers fostered with wild-type dams survive and develop normally, indicating that the defect lies in the ability of MMTV-*myc* or MTB/TOM mice to nurture their young and not with the pups themselves. Thus, deregulated MYC expression during pregnancy is both necessary and sufficient to block postpartum lactation and mimics the phenotype observed in MMTV-*myc* mice.

During normal mammary gland development, Myc expression peaks at day 6 of pregnancy and remains elevated during the proliferative phase of lobuloalveolar development through day 12.5 of pregnancy (Fig. 1A). MYC expression levels subsequently return to baseline by day 18.5 of pregnancy. To identify a developmental window within pregnancy that is disrupted by aberrant Myc activity, we induced MYC expression for discrete periods during pregnancy and assayed pup viability at 24 hours postpartum. Using this approach, we identified a period from D12.5-D15.5 of pregnancy during which MYC expression is necessary and sufficient to prevent postpartum lactation, and which results in the death of all pups within 24 hours of parturition (Fig. 1B). Restriction of MYC expression to 24 or 48 hours within this developmental window was not sufficient to cause pup death postpartum, although it did have a modest inhibitory effect on postpartum pup growth. Expression of MYC for extended periods of time from early to mid-pregnancy (D0.5-D11.5) resulted only in mild effects on postpartum pup growth and had no impact on pup survival. Remarkably, expression of MYC during late (D15.5-D18.5) pregnancy had no impact on pup survival or postpartum growth. Thus, we conclude that the 72-hour period from day 12.5 to 15.5 of pregnancy represents a discrete developmental window of susceptibility to the effects of MYC; overexpression of MYC during this period is both necessary and sufficient to block postpartum lactation and cause pup death.

Fig. 2. MMTV-*myc* and MTB/TOM mice exhibit similar mammary gland phenotypes during pregnancy. Hematoxylin and Eosin-stained number 4 mammary gland sections from FVB wild-type, MMTV-*myc* and MTB/TOM mice at D18.5 of pregnancy. MTB/TOM mice were induced with doxycycline from D0.5-D18.5 of pregnancy. Results are representative of sections from three animals per group.



MMTV-*myc* and MTB/TOM mice exhibit similar pregnancy phenotypes

To determine the mechanism by which MYC expression during pregnancy inhibits postpartum lactation, we assessed the effects of MYC on mammary development at the morphological level. Rather than blocking or otherwise delaying lobuloalveolar development, expression of Myc throughout pregnancy in either MMTV-*myc* or MTB/TOM mice resulted in the production of luminal secretions and precocious alveolar distension by D18.5 of pregnancy (Fig. 2). Importantly, the morphological phenotype of the inducible MTB/TOM model and the constitutive MMTV-*myc* model were comparable histologically, indicating that the mechanism accounting for the lactation defect in each model is likely similar. These results suggested the counterintuitive possibility that expression of MYC throughout pregnancy may block postpartum lactation by promoting precocious lactation during pregnancy.

MYC expression from D12.5-D15.5 of pregnancy induces precocious lactation

To further investigate the mechanism of MYC-mediated inhibition of postpartum lactation, we assessed the morphological effects of MYC expression from D12.5-D15.5 of pregnancy. Induction of MYC beginning at D12.5 of pregnancy resulted in precocious lobuloalveolar development, alveolar distention and secretion of eosinophilic material into the alveolar lumen by D15.5 (Fig. 3, compare A-D with E-H). Moreover, morphological differentiation of the gland continued even after animals were withdrawn from doxycycline and MYC transgene expression returned to uninduced levels (Fig. 3, compare I-K with M-O). By D18.5 of pregnancy, animals that had expressed the MYC transgene from D12.5-D15.5 of pregnancy exhibited engorged alveoli that were similar in morphological appearance to wild-type or uninduced glands during mid-lactation (Fig. 3K,S). Furthermore, precociously distended alveoli that had developed in mice induced to express MYC from D12.5-15.5 of pregnancy collapsed at day 1 postpartum (Fig. 3L), suggesting that MYC overexpression induces precocious lactation followed by premature mammary involution.

To investigate whether MYC expression from D12.5-D15.5 of pregnancy promotes precocious lactation at the molecular level, we assessed expression of the lactation-specific mRNAs, epsilon-casein (*Csnd*) (Hennighausen and Sippel, 1982) and prolactin-inducible protein (*Pip*) (Myal et al., 1991) as a function of MYC transgene expression and expression of endogenous *Myc*. Induction of MTB/TOM mice resulted in detectable MYC transgene expression as well as repression of the endogenous *Myc* locus within 24 hours following doxycycline administration. Within 3 days of MYC induction, MTB/TOM mice exhibited a marked increase in expression of

both *Csmd* and *Pip* mRNA compared with doxycycline-induced MTB controls (Fig. 4A). Following doxycycline withdrawal, *MYC* transgene expression was undetectable at the mRNA level within 24 hours, although endogenous *Myc* levels remained repressed for an additional 48 hours, indicating persistent activity of MYC at the protein level (Facchini et al., 1997). Notably, expression of *Csmd* and *Pip* continued to rise following doxycycline withdrawal through D18.5 of pregnancy. MTB/TOM mice induced to express MYC from D12.5-D15.5 of pregnancy also exhibited expression of the milk proteins α -casein and β -casein to late pregnancy levels by D15.5 of pregnancy (Fig. 4B). These data demonstrate that expression of MYC in the mammary gland from D12.5-D15.5 of pregnancy results in precocious lactation at both the molecular and morphological level.

MYC expression during pregnancy promotes aberrant mammary epithelial proliferation

Normal differentiation of the mammary gland during

pregnancy begins with proliferation of alveolar cells followed by differentiation of the alveolar epithelium to a non-proliferating secretory state (Briskin, 2002; Neville et al., 2002). We investigated the hypothesis that MYC promotes precocious mammary differentiation by stimulating aberrant proliferation of mammary alveolar epithelial cells. Although there was no significant difference ($P=0.40$) in the percentage of BrdU-positive cells between uninduced MTB ($6.0\pm 0.86\%$) and MTB/TOM ($7.50\pm 1.4\%$) mammary glands at D12.5 of pregnancy, BrdU immunohistochemistry (IHC) revealed a tenfold increase ($P=0.0004$) in the rate of mammary epithelial proliferation in MTB/TOM ($39.8\pm 6.83\%$) compared with MTB ($3.6\pm 0.59\%$) glands within 24 hours of doxycycline treatment (Fig. 5A). Consistent with this, Northern analysis demonstrated a clear increase in the mammary expression of cyclin A mRNA in MYC-expressing mammary glands (Fig. 5B). Within 1 day of doxycycline withdrawal, cyclin A mRNA expression and mammary epithelial proliferation rates in MTB/TOM glands ($7.60\pm 0.65\%$) had nearly returned to the

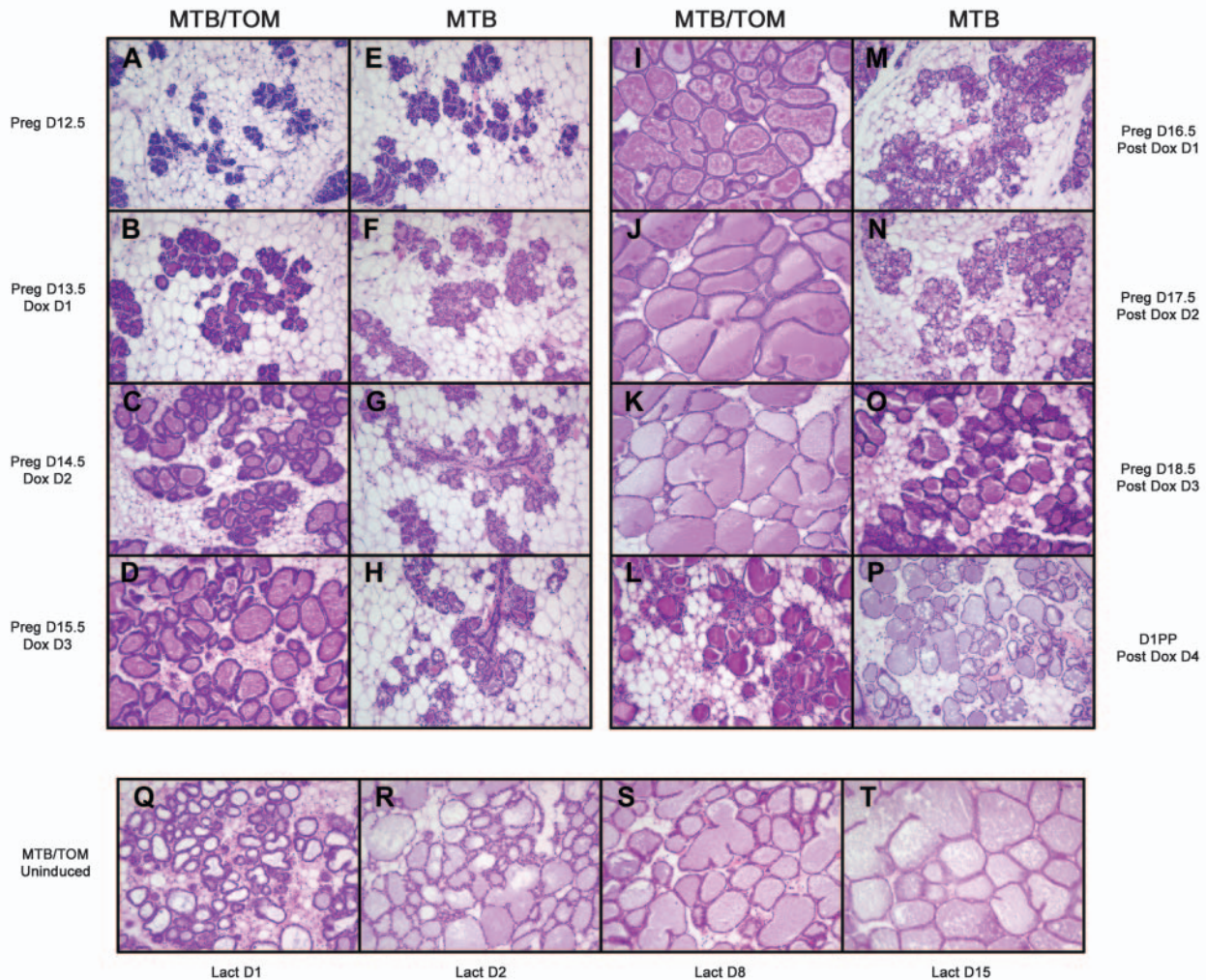
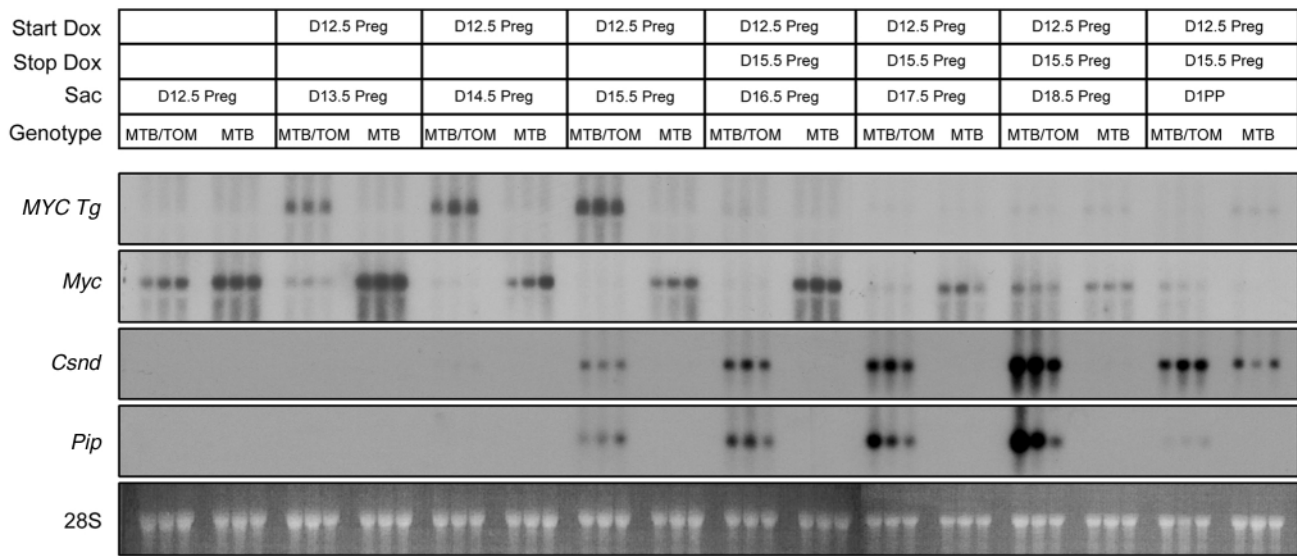
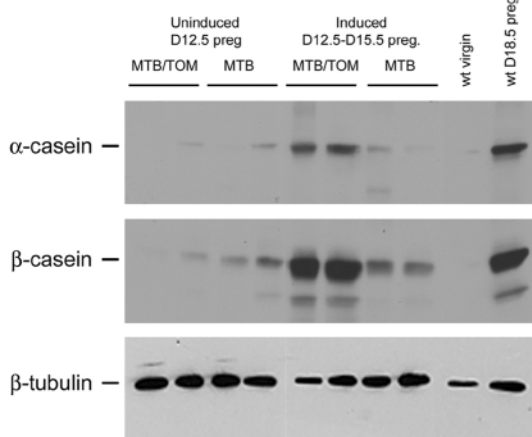


Fig. 3. Mammary epithelial expression of MYC from D12.5-D15.5 of pregnancy promotes morphological changes consistent with precocious lactation. Hematoxylin and Eosin staining of number four mammary gland histological sections from MTB and MTB/TOM mice induced with doxycycline from D12.5-D15.5 of pregnancy and harvested at 1-day increments from D12.5 of pregnancy through day 1 postpartum (D1PP) (A-P). Uninduced MTB/TOM glands harvested at increasing intervals during lactation are also shown (Q-T). Results are representative of sections from three animals per group.

A



B



level of MTB controls ($4.1 \pm 0.77\%$; $P=0.0057$). Thus, MYC-induced aberrant mammary epithelial proliferation may be the initial event in accelerating lobuloalveolar development and promoting premature lactation in MTB/TOM mice.

MYC induces premature Stat5 activation

Proliferation of alveolar epithelial cells and progression to a lactogenic state is mediated in part by activation of the prolactin receptor-Jak2-Stat5 signaling pathway (Hennighausen et al., 1997; Hynes et al., 1997; Kelly et al., 2002). This signaling pathway results in activation and tyrosine phosphorylation of residues Y694 and Y699 of Stat5a and Stat5b, respectively (Liu et al., 1996). During normal mammary gland development, Stat5 activation increases late in pregnancy in response to increased prolactin signaling (Liu et al., 1996). Induction of the *MYC* transgene from D12.5-D15.5 of pregnancy, however, resulted in a marked and premature increase in Stat5a/b phosphorylation beginning at 24 hours and peaking 48 hours after MYC induction at D14.5 of pregnancy (Fig. 6). Phospho-Stat5 levels remained elevated throughout

Fig. 4. Mammary epithelial expression of MYC from D12.5-D15.5 of pregnancy promotes precocious expression of markers of lactation. (A) Northern hybridization analysis of total RNA extracted from MTB and MTB/TOM mouse mammary glands induced with doxycycline from D12.5-D15.5 of pregnancy and harvested at 1-day increments from D12.5 pregnancy through D1PP. Northern blots were hybridized with probes specific to the human *MYC* transgene (*MYC Tg*), endogenous mouse *Myc*, *Csnd* and prolactin-inducible protein (*Pip*). Ethidium bromide staining of 28S RNA is shown as a loading control. (B) Western blot analysis of milk protein expression. Mammary protein lysates from uninduced MTB and MTB/TOM mice at D12.5 pregnancy and MTB and MTB/TOM mice induced from D12.5-D15.5 of pregnancy were separated by SDS-PAGE and transferred to nitrocellulose. Mammary lysates from FVB wild-type virgin mice and mice at D18.5 of pregnancy are included as controls. Membranes were probed with polyclonal antiserum specific to mouse milk proteins and β -tubulin. Expression of α -casein and β -casein are indicated.

the 72-hour period of MYC induction and returned to baseline within 24 hours after withdrawal of doxycycline treatment, paralleling *MYC* transgene levels. These findings suggest that MYC induced precocious lactation occurs through premature Stat5 hyperactivation.

MYC-induced precocious lactation is followed by epithelial apoptosis

Interestingly, following the return of phospho-Stat5 to baseline levels at D16.5 in MTB/TOM mice induced with doxycycline from D12.5-D15.5 of pregnancy, we observed a precipitous drop in Stat5a/b tyrosine phosphorylation to levels below those of MTB controls that persisted from D17.5 of pregnancy through parturition. This suppression of phospho-Stat5 levels was reminiscent of the decrease in Stat5 activation observed at the onset mammary involution (Chapman et al., 1999). During normal mammary development, involution occurs following the cessation of suckling by pups and is accompanied by widespread apoptosis of mammary alveolar epithelial cells that is triggered by milk stasis (Quarrie et al., 1996). Because milk

produced as a consequence of MYC induction during pregnancy would have no outlet, we considered the possibility that Myc-induced precocious lactation might lead to milk stasis-induced precocious alveolar apoptosis.

To investigate patterns of apoptosis induced in the mammary gland by MYC activation, we performed TUNEL analysis at increasing intervals following doxycycline induction of MTB/TOM mice. Uninduced MTB and MTB/TOM mice at D12.5 of pregnancy exhibited equivalent rates of apoptosis (Fig. 7A,E) (MTB/TOM $3.2 \pm 1.1\%$ compared with MTB $2.9 \pm 0.67\%$; $P=0.82$). Consistent with the known pro-apoptotic effects of MYC, induction of MYC from D12.5-D15.5 of pregnancy resulted in a marked increase in the proportion of TUNEL-positive mammary epithelial cells within 24 hours of doxycycline treatment (Fig. 7B,F; MTB/TOM $4.1 \pm 0.35\%$ compared with MTB $1.0 \pm 0.39\%$; $P=0.0002$). This increased apoptotic rate persisted throughout the period of MYC induction (Fig. 7C,G; MTB/TOM $6.5 \pm 1.44\%$ compared with MTB $0.62 \pm 0.17\%$; $P=0.0023$) (Fig. 7D,H; MTB/TOM $4.3 \pm 0.60\%$ compared with MTB $1.00 \pm 0.45\%$; $P=0.0012$) and up until 1 day post-deinduction (Fig. 7I,M; MTB/TOM $12.6 \pm 1.90\%$ compared with MTB $1.44 \pm 0.62\%$; $P=0.0002$). By day 17.5 of pregnancy, 2 days post-doxycycline withdrawal, apoptotic rates in MTB/TOM mice (Fig. 7J; $0.53 \pm 0.36\%$) had returned to the level of MTB control mice (Fig. 7N; $0.16 \pm 0.16\%$; $P=0.38$). Intriguingly, at D18.5 of pregnancy, 3 days post-doxycycline withdrawal, apoptosis rates were again markedly elevated in MTB/TOM mice (Fig. 7K; $4.8 \pm 1.64\%$) compared with MTB controls (Fig. 7O; $0.07 \pm 0.07\%$; $P=0.017$), and remained elevated at D1PP (Fig. 7L,P; MTB/TOM $8.97 \pm 1.50\%$ compared with MTB $0.38 \pm 0.24\%$; $P=0.0002$).

This bimodal pattern of mammary epithelial apoptosis is consistent with two distinct phases. High apoptosis rates observed from D13.5-D16.5 of pregnancy are consistent with the known pro-apoptotic effects of aberrant MYC activation and are probably a direct consequence of its expression. The second wave of apoptosis induction in this system, however, occurs in the absence of MYC pathway activation and could occur as a consequence of milk-stasis-induced involution.

MYC promotes premature activation of Stat3 and upregulation of Tgfb3

Mammary involution at the time of pup weaning is triggered by milk stasis and occurs as a consequence of activation of the Stat3 and Tgfb3 signaling pathways (Chapman et al., 1999; Humphreys et al., 2002). Given that MYC-induced precocious

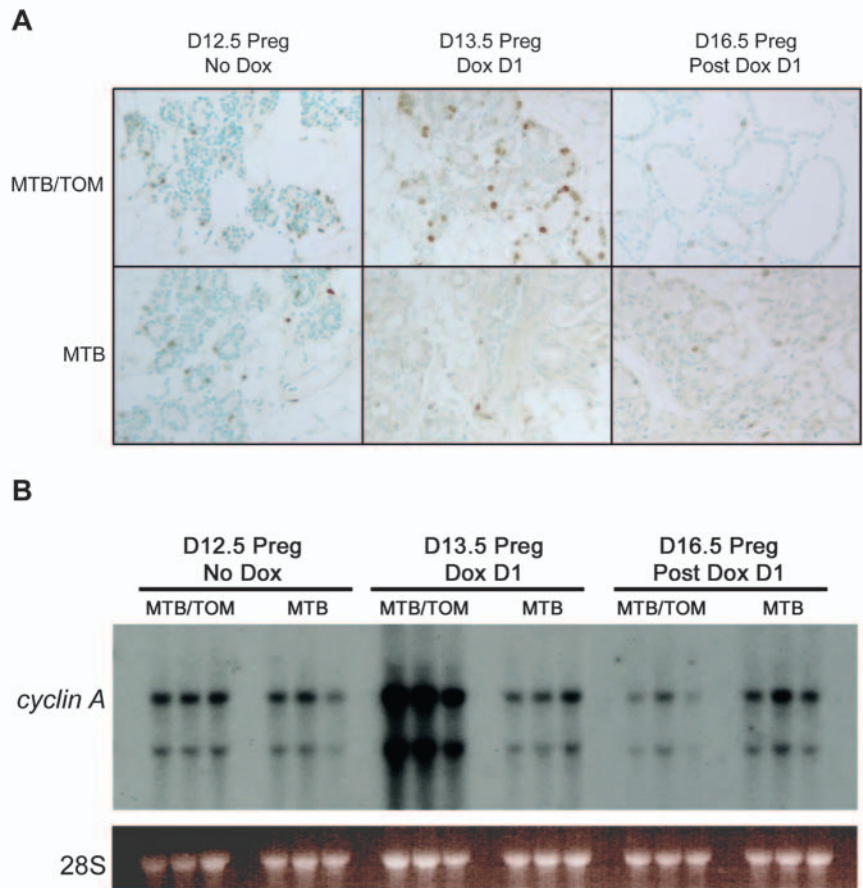
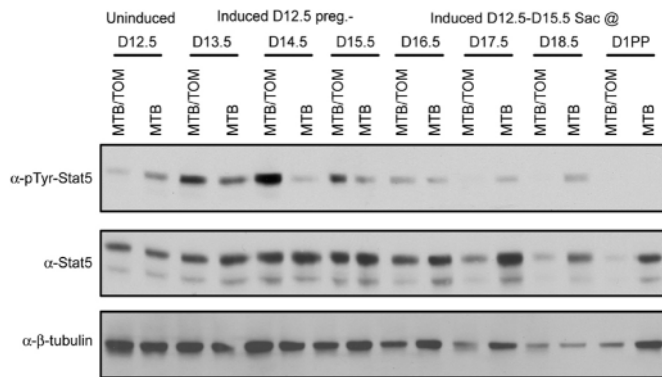


Fig. 5. MYC expression during pregnancy induces abnormal mammary epithelial proliferation. (A) BrdU immunohistochemistry of uninduced MTB and MTB/TOM mice at D12.5 of pregnancy; MTB and MTB/TOM mice induced with doxycycline from D12.5-D13.5 sacrificed at D13.5 of pregnancy; and MTB and MTB/TOM mice induced from D12.5-D15.5 and sacrificed at D16.5 of pregnancy. Mice were injected i.p. with 50 mg/kg BrdU 2 hours prior to sacrifice. Histological sections from number four mammary glands were assessed for incorporation of BrdU by IHC analysis. Results are representative of three animals per group. (B) Northern hybridization analysis of total RNA extracted from the mammary glands of MTB and MTB/TOM mice induced with doxycycline as indicated. Total RNA was separated by gel electrophoresis, transferred to nitrocellulose and hybridized with a radiolabeled probe specific to cyclin A mRNA. Ethidium bromide staining of 28S RNA is shown as a loading control.

lactation during pregnancy would be expected to result in milk stasis, we hypothesized that the second wave of apoptosis observed in MTB/TOM mice might occur via Stat3 and Tgfb3-mediated pathways. Consistent with this prediction, induction of MYC expression from D12.5-D15.5 of pregnancy in MTB/TOM mice resulted in a dramatic increase in the level of Stat3 Y705 phosphorylation in mammary tissue extracts beginning at D15.5 of pregnancy, peaking at D1PP at a level similar to that observed at day 2 of involution in wild-type mice (Fig. 8A).

In addition to Stat3, Tgfb3 is also an important regulator of the involution process (Nguyen and Pollard, 2000). Expression of *Tgfb3* mRNA as well as the *Tgfb* target gene, *Serpine1* (Stampfer et al., 1993), were sharply upregulated at D18.5 of pregnancy and D1PP in MTB/TOM mice induced with doxycycline from D12.5-15.5 of pregnancy (Fig. 8B). In aggregate, our results suggest that MYC-induced precocious



lactation results in premature involution of the gland mediated by milk stasis-induced activation of the Stat3 and Tgfb3 signaling pathways.

MYC-induced Stat5 activation and precocious lactation are developmental stage-specific

To determine whether MYC-induced precocious lactation is dependent on the developmental stage of the mammary gland at the time at which MYC is expressed, we compared the effects of MYC induction from D12.5-D15.5 of pregnancy

Fig. 6. Mammary epithelial expression of MYC from D12.5-D15.5 of pregnancy promotes premature Stat5 hyperactivation. MTB and MTB/TOM mice induced with doxycycline from D12.5-D15.5 were sacrificed at 24-hour intervals from D12.5 pregnancy-D1PP. Mammary protein lysates were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were probed with antibodies specific to Y694/Y699 phosphorylated Stat5a/b, total Stat5a/b, and β -tubulin. Results are representative of experiments performed on two separate sets of animals.

with those from D6.5-D9.5 of pregnancy. In contrast to the precocious alveolar distension and luminal secretion observed when Myc was expressed from D12.5-D15.5 of pregnancy, mammary epithelial expression of Myc from D6.5-D9.5 of pregnancy resulted in no discernable increase in lobuloalveolar development within the mammary fat pad by D9.5 of pregnancy (Fig. 9A). Similarly, there were no detectable morphological differences between the mammary glands of doxycycline-induced MTB/TOM and MTB mice 3 days post-doxycycline induction at D12.5 of pregnancy (Fig. 9A).

MYC also exhibited a developmental stage-specific ability to induce molecular changes associated with precocious lactation. For example, MYC induction from D12.5-D15.5 of pregnancy was sufficient to induce *Csnd* and *Pip* expression, whereas MYC induction from D6.5-D9.5 of pregnancy was not

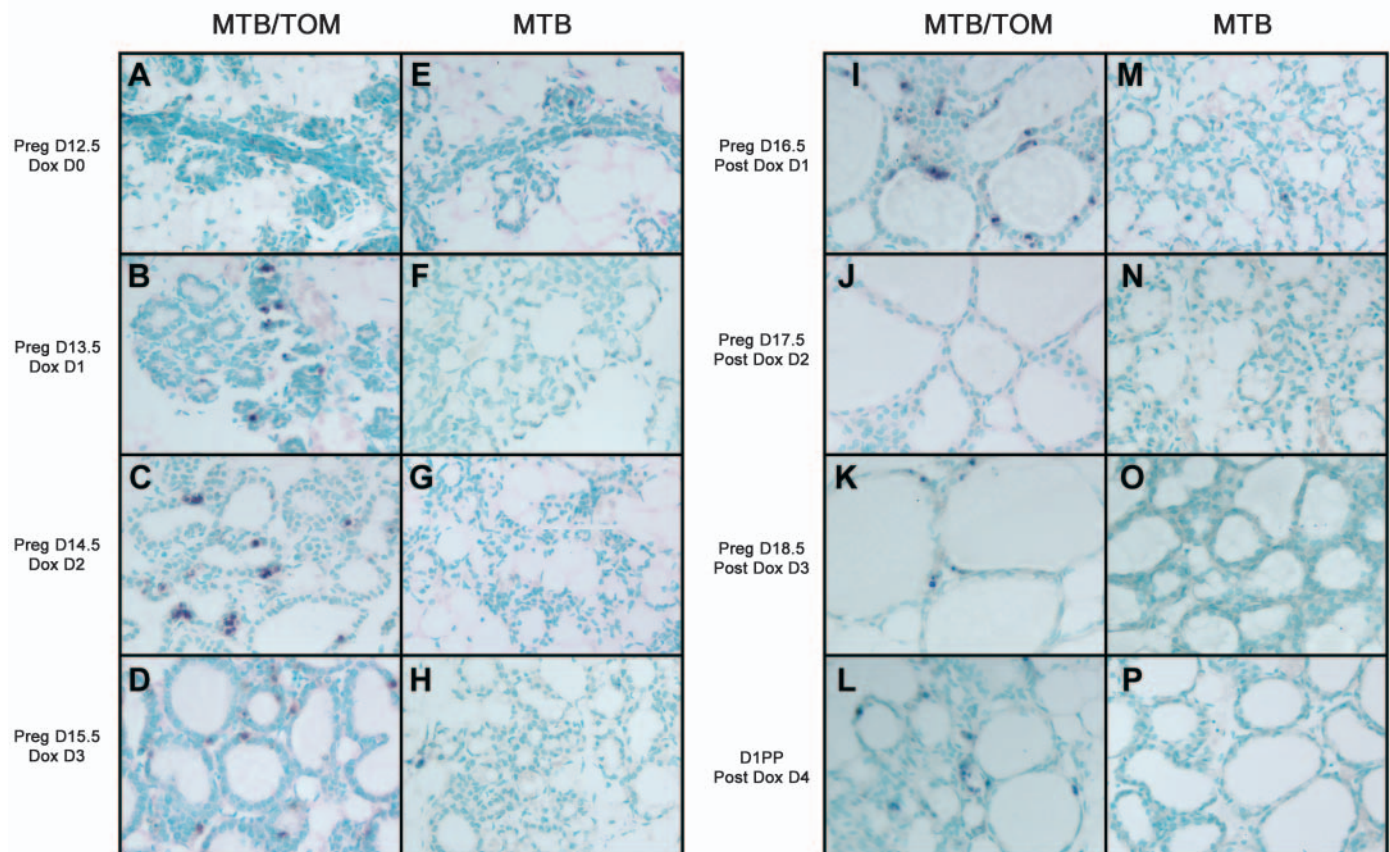


Fig. 7. Expression of MYC from D12.5-D15.5 of pregnancy promotes apoptosis of mammary epithelial cells. Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) analysis was performed using the Apoptag Peroxidase Kit on mammary histological sections from MTB/TOM (A-D) and MTB control mice (E-H) induced with doxycycline from D12.5-D15.5 of pregnancy. TUNEL analysis was also performed on mammary gland histological sections from MTB/TOM (I-L) and MTB mice (M-P) during the post-induction period from day 16.5 of pregnancy to day 1 postpartum (D1PP). Images are representative of three animals per group.

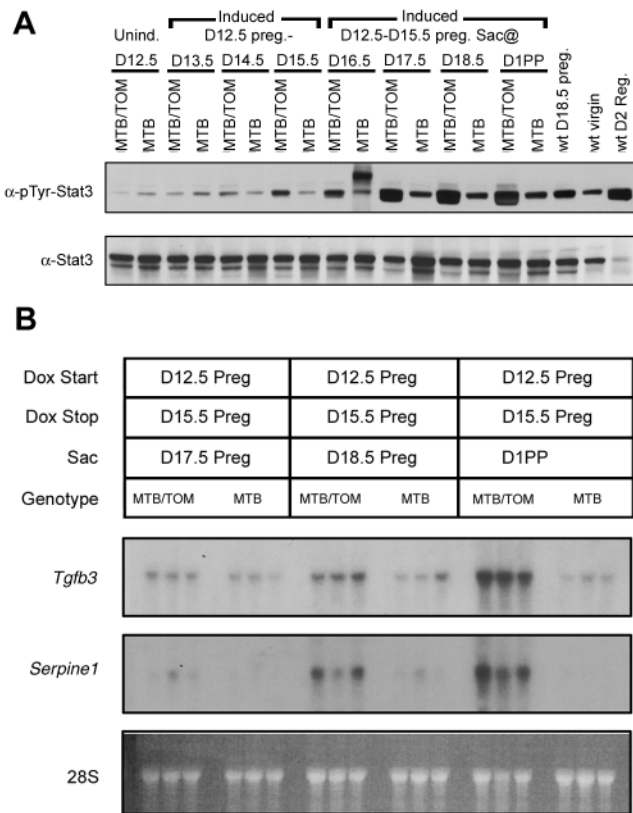


Fig. 8. MYC expression promotes activation of the Stat3 and *Tgfb3* involvement pathways. (A) The Y705-phosphorylation status of Stat3 was determined by western blot analysis. Mammary protein lysates from MTB and MTB/TOM mice induced with doxycycline from D12.5-D15.5 of pregnancy and sacrificed at 24-hour intervals from D12.5 of pregnancy through D1PP were separated by SDS-PAGE and transferred to nitrocellulose membranes. Lysates from FVB wild-type mice harvested during virgin development, D18.5 of pregnancy and D2 of regression were included as controls. Membranes were probed with antibodies specific to Y705-phosphorylated Stat3 and total Stat3. Results are representative of experiments performed on two separate sets of animals. (B) Total RNA was extracted from mammary glands of MTB and MTB/TOM animals induced from D12.5-D15.5 of pregnancy and sacrificed at D17.5-D1PP. Expression of *Tgfb3* mRNA and a direct *Tgfb3* target, *Serpine1*, were assessed by northern hybridization analysis with radiolabeled probes.

(Fig. 9B). Importantly, mRNA expression of the *MYC* transgene was equivalent in MTB/TOM mice induced from D6.5-D9.5 of pregnancy and those induced from D12.5-D15.5 of pregnancy. Moreover, expression of the *MYC* transcriptional targets, *Cdk4*, *Fbl* and *Shmt1* (D'Cruz et al., 2001; Haggerty et al., 2003), were all comparable in MTB/TOM mice induced during these same developmental windows (Fig. 9B). This indicates that although the extent of *MYC* pathway activation is similar in the mammary glands of mice induced from D6.5-D9.5 and D12.5-D15.5 of pregnancy, the consequences of *MYC* activation are not. This strongly suggests that the morphological and molecular differences observed when *MYC* is expressed from D6.5-D9.5 or D12.5-D15.5 of pregnancy are probably due to developmental stage-specific effects of *MYC* in the mammary gland, rather than

differences in *MYC* transgene expression or activity levels at the time of induction.

To determine whether *MYC*-induced Stat5 hyperactivation also occurs in a developmental stage-specific manner, we assessed Stat5a/b Y695/Y699 phosphorylation in response to *MYC* expression from D6.5-D9.5 of pregnancy. Induction of *MYC* in MTB/TOM mammary glands from D6.5-D7.5, D6.5-D8.5 or D6.5-D9.5 of pregnancy failed to result in a detectable difference in Stat5 tyrosine-phosphorylation as compared with MTB controls (Fig. 9C). This is in contrast to the elevated Stat5 tyrosine-phosphorylation observed when *MYC* was expressed from D12.5-D13.5, D12.5-D14.5 or D12.5-D15.5 of pregnancy (Fig. 9C). Though subtle, this effect has consistently been observed in multiple independent experiments. Thus, the developmental stage-specific ability of *MYC* to induce precocious lactation is tightly correlated with its ability to induce Stat5 activation.

MYC down-regulates caveolin 1 expression in a developmental stage-specific manner

Downregulation of negative regulators of the Prrl-Jak2-Stat5 signaling pathway have been proposed to play a vital role in the transition of the mammary gland from pregnancy to lactation. Genetic deletion of *Socs1* or *Cav1*, for example, have been shown to result in premature Stat5 activation and precocious lactation during pregnancy (Lindeman et al., 2001; Park et al., 2002). Interestingly, experiments in NIH3T3 and Rat1 cells have shown that *MYC* can directly repress *Cav1* transcription (Park et al., 2001; Timme et al., 2000). Therefore, we hypothesized that the developmental stage-specific ability of *MYC* to activate Stat5 might be due to the ability of *MYC* to downregulate *Cav1* expression in the mouse mammary gland in a developmental stage-specific manner.

Indeed, northern blot analysis demonstrated rapid downregulation of *Cav1* mRNA levels when *MYC* was induced from D12.5-D15.5, but not D6.5-D9.5, of pregnancy (Fig. 10A). By contrast, *MYC* did not downregulate expression of the suppressor of cytokine signaling gene, *Cish*, when induced from D6.5-D9.5 or D12.5-D15.5 of pregnancy (Fig. 10A). Western blot analysis demonstrated that *Cav1* protein expression was markedly downregulated following 48 hours of *MYC* induction from D12.5-D14.5, but not D6.5-D8.5, of pregnancy (Fig. 10B). Thus, the developmental stage-specific ability of *MYC* to downregulate mammary expression of *Cav1* in vivo is closely linked with its ability to promote Stat5 activation and precocious lactation.

Discussion

Breast cancer susceptibility has long been known to be markedly influenced by the timing of normal developmental events that occur over the course of a woman's lifetime. The molecular basis for this epidemiological observation, however, has remained obscure. Based on this, we hypothesized that the effects of oncogene activation might be modulated by the developmental state of the mammary gland. That is, the consequences of activating the same oncogene at the same level could differ depending upon the developmental state of the gland at the time of oncogene activation. Using a conditional transgenic model for *MYC* expression, we have confirmed this

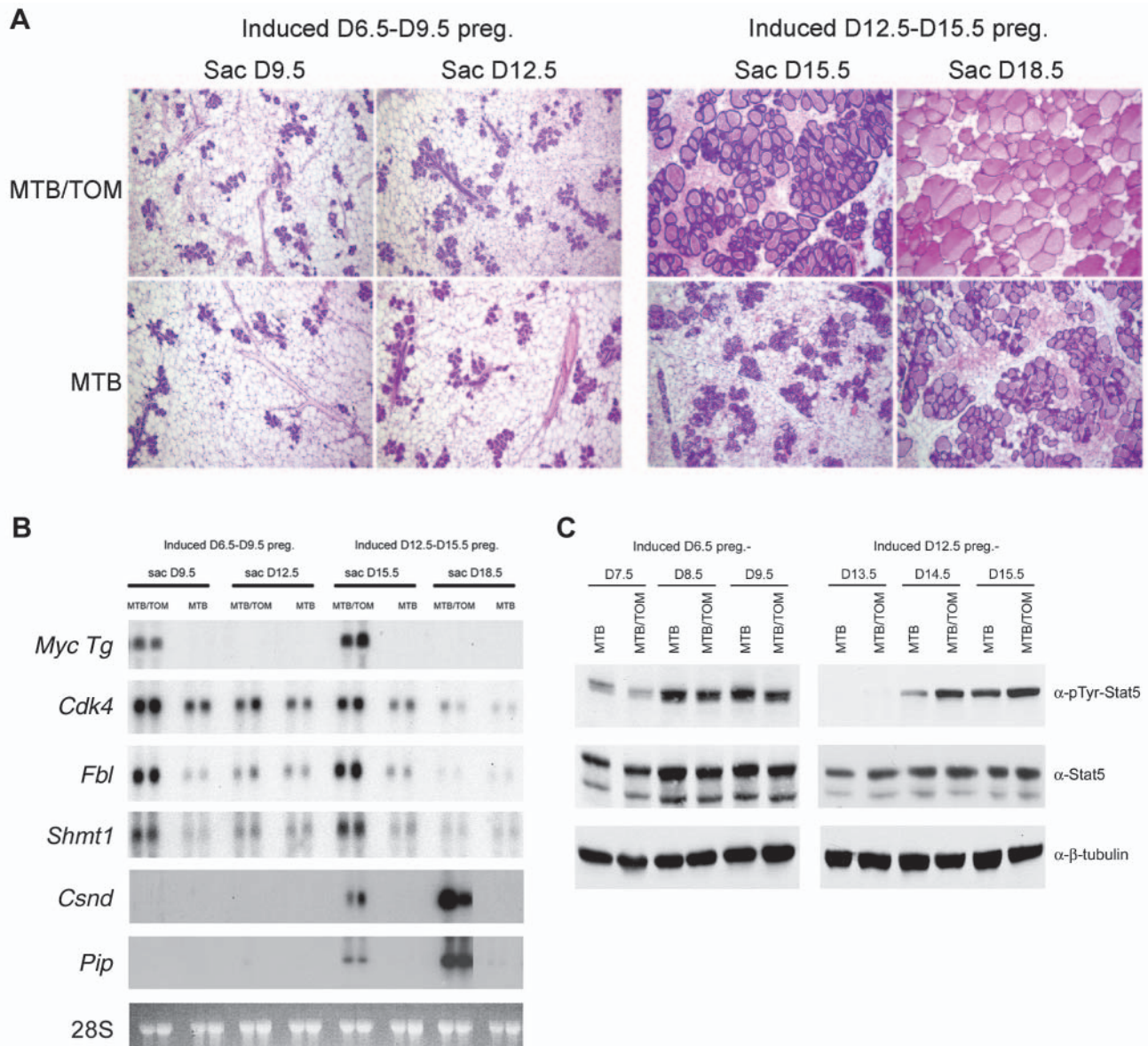


Fig. 9. MYC-induced Stat5 activation and precocious lactation are specific to MYC induction from D12.5-D15.5 of pregnancy. (A) Hematoxylin and Eosin staining of mammary histological sections from MTB and MTB/TOM mice induced with doxycycline from D6.5-D9.5 of pregnancy and sacrificed (sac) at D9.5 or D12.5 of pregnancy, as well as from mice induced from D12.5-D15.5 and sacrificed at D15.5 or D18.5 of pregnancy. Results are representative of experiments performed on three separate sets of animals. (B) Expression of the *Myc* transgene, targets of MYC transcriptional activation *Cdk4*, *Fbl* and *Shmt1*, and the lactation markers *Csnd* and *Pip* were determined by northern hybridization analysis. Total mammary RNA was prepared from MTB and MTB/TOM mice induced from D6.5-D9.5 or D12.5-D15.5 of pregnancy. (C) Western blot analysis of Stat5a/b Y694/Y699 phosphorylation. Mammary protein extracts were prepared from MTB and MTB/TOM mice induced from D6.5-D7.5, D6.5-D8.5 and D6.5-D9.5 or D12.5-D13.5, D12.5-D14.5 and D12.5-D15.5 of pregnancy. Proteins were separated by SDS-PAGE and blotted to nitrocellulose membranes. Blots were probed with antibodies specific to Y694/Y699 phosphorylated Stat5a/b, total Stat5a/b, and β -tubulin. Results are representative of experiments performed on two separate sets of animals.

prediction by identifying a developmental stage-specific effect of MYC in the mammary gland during pregnancy.

We have demonstrated that the ability of MYC to inhibit postpartum lactation is entirely due to its activation within a specific 72-hour window during mid-pregnancy, and that this developmental stage-specific window of susceptibility correlates tightly with the ability of MYC to downregulate *Cav1* and activate Stat5 in a developmental stage-specific manner. MYC expression during a defined 72 hour window in

mid-pregnancy is both necessary and sufficient to inhibit postpartum lactation. Surprisingly, MYC expression during this period does not block postpartum lactation by inhibiting mammary epithelial differentiation, but rather by accelerating mammary epithelial differentiation and inducing mice to lactate during pregnancy. MYC-induced precocious lactation results in milk stasis, aberrant Stat3 and Tgfb3 activation, and premature involution of the gland, thereby accounting for the inability of mice to lactate postpartum. These findings provide

Development and disease

new insights into the mechanism by which the developmental state of the mammary gland modulates its response to oncogene exposure.

In the vast majority of cell types studied, deregulated MYC expression is incompatible with the terminally differentiated state (Brandvold et al., 2001; Freytag, 1988; Heath et al., 2000; Miner and Wold, 1991). This is typically ascribed to the role of MYC as a potent promoter of cellular proliferation through its ability to transcriptionally activate and repress genes vital to cell cycle regulation (Bouchard et al., 1998). Contrary to this paradigm, however, are studies by Gandarillas and Watt, demonstrating that Myc expression in epidermal stem cells actually promotes terminal differentiation by driving cells into the transit amplifying compartment (Gandarillas and Watt, 1997). These and other observations suggest the intriguing possibility that in cell types in which proliferation is an important aspect of the differentiation process, Myc may actually promote, or even be required for terminal differentiation (Arnold and Watt, 2001; Battaglini et al., 2002; Gandarillas and Watt, 1997).

In light of the above findings, the ability of MYC to promote rather than inhibit mammary epithelial differentiation may stem from the fact that normal differentiation of the mammary gland proceeds through a series of proliferative steps. During early pregnancy, side-branches arise from existing ducts as a subset of ductal epithelial cells proliferate in response to progesterone (Briskin, 2002). As pregnancy progresses, continuous proliferation of side branches gives rise to alveolar clusters in a Prl-dependent mechanism known as lobuloalveolar development (Briskin, 2002). It is only after lobuloalveolar development that Prl-dependent lactogenesis can occur (Neville et al., 2002). We have shown that when expressed in the mammary epithelium from D12.5-D15.5 of pregnancy, MYC induces proliferation and accelerates lobuloalveolar hyperplasia. By accelerating lobuloalveolar development during pregnancy, we speculate that MYC brings the mammary epithelium to a state in which it is competent to undergo precocious terminal differentiation.

Stat5 plays a vital role in mammary development, as demonstrated by the fact that homozygous deletion of *Stat5a* and *Stat5b* inhibits both lobuloalveolar development and lactation (Miyoshi et al., 2001; Teglund et al., 1998). Downstream of Prlr, Stat5a and Stat5b are activated by Jak2 tyrosine kinase-mediated phosphorylation of residues Y694 and Y699, respectively. This event is tightly linked to mammary differentiation and lactation, peaking late in pregnancy during normal mammary gland development (Liu et al., 1996). Expression of MYC from D12.5-15.5 of pregnancy results in a striking shift in the peak of Stat5 tyrosine-phosphorylation from D18.5 to D14.5 of pregnancy. The tight link between Stat5 hyperactivation and lactation strongly suggests that this is the mechanism by which MYC promotes precocious lactation.

Though Prolactin-receptor binding and activation provide a Stat5 activating signal, negative regulation of Prolactin-receptor-Stat5 signaling pathways plays an equally important role in the regulation of Stat5 activity. In particular, the suppressor of cytokine signaling (SOCS) family of molecules, as well as the scaffolding protein, Cav1, have emerged as important negative regulators of cytokine-receptor-mediated Stat5 activation (Kile and Alexander, 2001).

Developmental stage-specific effects of MYC 1157

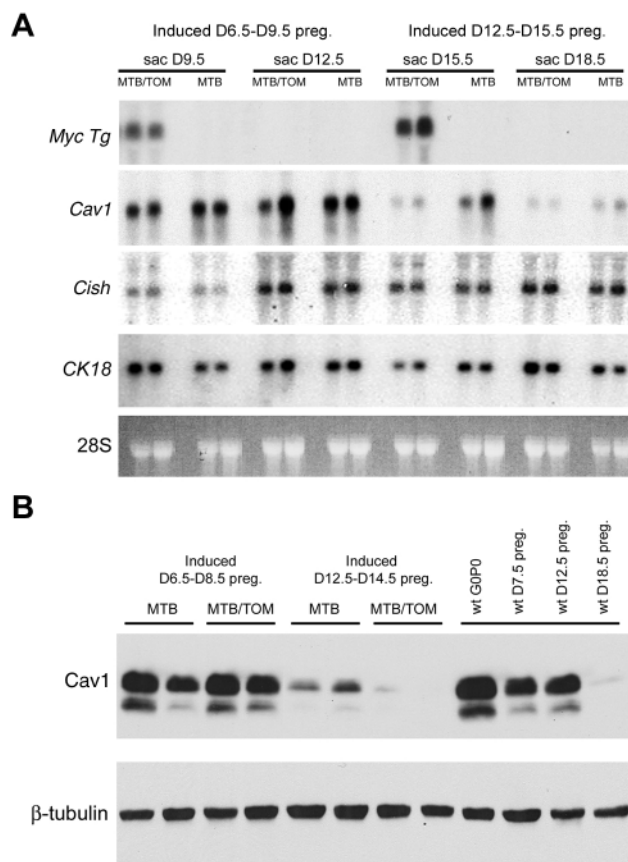


Fig. 10. MYC downregulates mammary expression of Cav1 in a developmental stage-specific manner. (A) Northern hybridization analysis. Total RNA was extracted from the mammary glands of MTB and MTB/TOM mice induced with doxycycline from D6.5-D9.5 of pregnancy and sacrificed at D9.5 or D12.5 of pregnancy, as well as from MTB and MTB/TOM mice induced from D12.5-D15.5 and sacrificed at D15.5 or D18.5 of pregnancy. Total RNA was subject to gel electrophoresis and blotted to nitrocellulose membranes. Blots were probed with radiolabeled cDNA fragments specific to the human MYC transgene, as well as mouse *Cav1*, *Cish* and *CK18*. Ethidium bromide staining of 28S RNA is shown as a loading control. (B) Western blot analysis of Cav1 expression. Mammary protein lysates from MTB and MTB/TOM mice induced from D6.5-D8.5 or D12.5-D14.5 of pregnancy were separated by SDS-PAGE and blotted to nitrocellulose. FVB wild-type mammary lysates from virgin, D7.5, D12.5 and D18.5 of pregnancy were included as controls. Blots were probed with antibodies specific to Cav1 and β -tubulin.

Cav1 negatively regulates Prlr-Jak2-Stat5 signaling in the mammary gland and plays an essential role in the transition from pregnancy to lactation. This is convincingly demonstrated by the fact that *Cav1*^{-/-} mice exhibit increased Jak2 kinase activity, hyperactivation of Stat5 and precocious lactation during pregnancy (Park et al., 2002). Indeed, mammary expression of *Cav1* mRNA is downregulated during late pregnancy and has been proposed to be the impetus for the transition of the mammary gland to a lactogenic state (Park et al., 2002). Intriguingly, reports by Lisanti et al. have identified *Cav1* as a direct target of Myc-mediated transcriptional repression in vitro (Park et al., 2001). We have confirmed that

MYC expression for as little as 48 hours in vivo, from D12.5-D14.5 of pregnancy, results in the dramatic downregulation of Cav1 expression in the mammary gland. Though it cannot be ruled out that repression of Cav1 expression is a consequence of Myc-induced precocious lactation and not the cause, our data support a model in which MYC-mediated repression of Cav1 expression results in upregulated Jak2 kinase activity, premature Stat5 hyperactivation and precocious lactation.

We have demonstrated that Stat3 activation, downregulation of Stat5 activity, and upregulation of *Tgfb3* expression occur shortly after induction of the precocious lactogenic state engendered in MTB/TOM mice by expression of MYC from D12.5-D15.5 of pregnancy. These molecular changes parallel events observed during the early stages of mammary involution in response to milk stasis. Normal involution occurs in two distinct phases. Stage 1 is reversible and initiated within hours of forced weaning or teat sealing. This stage is characterized by a rapid increase in *Tgfb3* expression (Nguyen and Pollard, 2000), activation of Stat3, and decreased activation of Stat5 (Chapman et al., 1999). Importantly, both *Tgfb3* and *Stat3* are required for normal involution to occur, as is downregulation of Stat5 activity (Chapman et al., 1999; Iavnilovitch et al., 2002; Nguyen and Pollard, 2000). These molecular changes precede apoptosis of alveolar cells, which is initiated within 24 and 48 hours of milk stasis (Quarrie et al., 1996). The second stage of involution, which begins between involution day 3 and day 4, is irreversible and is accompanied by tissue restructuring in the form of alveolar collapse and replacement with adipose tissue (Quarrie et al., 1996). We observe similar molecular, cellular and morphological alterations in MTB/TOM mouse mammary glands that have been induced to undergo precocious lactation. Taken together, our findings support a model in which MYC expression from D12.5-D15.5 of pregnancy induces precocious lactation, which results in milk stasis, premature involution and the inability of mice to nurse their pups postpartum.

The observation that MYC activates the established anti-apoptotic survival factor Stat5 has important implications for MYC-induced mammary tumorigenesis. Aberrant MYC expression induces apoptosis in a number of cell types, including mammary epithelial cells (D'Cruz et al., 2001). Interestingly, the latency for MYC-induced tumor formation is reduced dramatically in the setting of concurrent activation of anti-apoptotic pathways, such as the Bcl2 family member Bcl-x_L (Pelengaris et al., 2002). Our findings provide evidence that deregulated MYC expression upregulates a known anti-apoptotic survival pathway in vivo. It is tempting to speculate that MYC may require activation of Stat5 as a mechanism to avoid apoptosis in the processes of tumor initiation and progression. Recent genetic studies have suggested a similar role for Stat5 in TGF α and SV40 large T antigen-induced mammary tumorigenesis (Humphreys and Hennighausen, 1999; Ren et al., 2002). A role for the Prlr and Stat5 in MYC-induced mammary tumorigenesis has yet to be determined. Genetic studies with *Prlr*-null and *Stat5a/b*-null mice should help elucidate the function of this important mammary developmental signaling pathway in MYC-induced mammary tumor formation.

The developmental stage-specific manner in which MYC downregulates Cav1, drives premature Stat5 hyperactivation, and induces precocious lactation has important implications for

our understanding of the developmental stage-specific effects of oncogene action. Previously, in vitro studies have suggested that the effects of deregulated MYC expression may be dependent on the cellular context in which it is expressed. For example, Evan et al. have demonstrated that access to serum determines whether Rat1 fibroblasts proliferate or undergo apoptosis in response to MYC expression (Evan et al., 1992). This suggests that cellular access to nutrients and growth factors may determine the consequence of MYC activation. The effects of aberrant MYC expression have also been shown to vary with cell type. Expression of MYC in suprabasal keratinocytes or lymphocytes drives proliferation, but results in little detectable apoptosis (Felsher and Bishop, 1999; Pelengaris et al., 1999). By contrast, targeted MYC expression in pancreatic β -cells results in overwhelming apoptosis with little detectable proliferation (Pelengaris et al., 2002). Our results indicate that the developmental state of a cell at the time of MYC exposure plays a crucial role in determining the biological effects of aberrant MYC activation. This is the first known developmental stage-specific effect of aberrant oncogene activation and suggests that MYC-induced mammary tumorigenesis may be similarly affected by the mammary developmental stage at which this oncogene is expressed.

This research was supported in part by NIH grants CA94393, CA92910 and CA93719 from the National Cancer Institute; by US Army Breast Cancer Research Program grant DAMD17-03-1-0345 (C.M.B.); and by a grant from the Susan G. Komen Breast Cancer Foundation.

References

- Andres, A. C., van der Valk, M. A., Schonenberger, C. A., Fluckiger, F., LeMeur, M., Gerlinger, P. and Groner, B. (1988). Ha-ras and c-myc oncogene expression interferes with morphological and functional differentiation of mammary epithelial cells in single and double transgenic mice. *Genes Dev.* **2**, 1486-1495.
- Arnold, I. and Watt, F. M. (2001). c-Myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny. *Curr. Biol.* **11**, 558-568.
- Askew, D. S., Ashmun, R. A., Simmons, B. C. and Cleveland, J. L. (1991). Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* **6**, 1915-1922.
- Bain, C., Willett, W., Rosner, B., Speizer, F. E., Belanger, C. and Hennekens, C. H. (1981). Early age at first birth and decreased risk of breast cancer. *Am. J. Epidemiol.* **114**, 705-709.
- Battaglino, R., Kim, D., Fu, J., Vaage, B., Fu, X. Y. and Stashenko, P. (2002). c-myc is required for osteoclast differentiation. *J. Bone Miner. Res.* **17**, 763-773.
- Berns, E. M., Klijn, J. G., Smid, M., van Staveren, I. L., Look, M. P., van Putten, W. L. and Foekens, J. A. (1996). TP53 and MYC gene alterations independently predict poor prognosis in breast cancer patients. *Genes Chromosomes Cancer* **16**, 170-179.
- Boice, J. D., Jr, Preston, D., Davis, F. G. and Monson, R. R. (1991). Frequent chest X-ray fluoroscopy and breast cancer incidence among tuberculosis patients in Massachusetts. *Radiat. Res.* **125**, 214-222.
- Bouchard, C., Staller, P. and Eilers, M. (1998). Control of cell proliferation by Myc. *Trends Cell Biol.* **8**, 202-206.
- Brandvold, K. A., Ewert, D. L., Kent, S. C., Neiman, P. and Ruddell, A. (2001). Blocked B cell differentiation and emigration support the early growth of Myc-induced lymphomas. *Oncogene* **20**, 3226-3234.
- Brisken, C. (2002). Hormonal control of alveolar development and its implications for breast carcinogenesis. *J. Mammary Gland Biol. Neoplasia* **7**, 39-48.
- Chapman, R. S., Lourenco, P. C., Tonner, E., Flint, D. J., Selbert, S., Takeda, K., Akira, S., Clarke, A. R. and Watson, C. J. (1999).

- Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev.* **13**, 2604-2616.
- Chrzan, P., Skokowski, J., Karmolinski, A. and Pawelczyk, T.** (2001). Amplification of c-myc gene and overexpression of c-Myc protein in breast cancer and adjacent non-neoplastic tissue. *Clin. Biochem.* **34**, 557-562.
- Coppola, J. A. and Cole, M. D.** (1986). Constitutive c-myc oncogene expression blocks mouse erythroleukaemia cell differentiation but not commitment. *Nature* **320**, 760-763.
- D'Cruz, C. M., Gunther, E. J., Boxer, R. B., Hartman, J. L., Sintasath, L., Moody, S. E., Cox, J. D., Ha, S. I., Belka, G. K., Golant, A., Cardiff, R. D. and Chodosh, L. A.** (2001). c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations. *Nat. Med.* **7**, 235-239.
- Deming, S. L., Nass, S. J., Dickson, R. B. and Trock, B. J.** (2000). C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. *Br. J. Cancer* **83**, 1688-1695.
- Dores, G. M., Metayer, C., Curtis, R. E., Lynch, C. F., Clarke, E. A., Glimelius, B., Storm, H., Pukkala, E., van Leeuwen, F. E., Holowaty, E. J. et al.** (2002). Second malignant neoplasms among long-term survivors of Hodgkin's disease: a population-based evaluation over 25 years. *J. Clin. Oncol.* **20**, 3484-3494.
- Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z. and Hancock, D. C.** (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**, 119-128.
- Facchini, L. M., Chen, S., Marhin, W. W., Lear, J. N. and Penn, L. Z.** (1997). The Myc negative autoregulation mechanism requires Myc-Max association and involves the c-myc P2 minimal promoter. *Mol. Cell Biol.* **17**, 100-114.
- Felsher, D. W. and Bishop, J. M.** (1999). Reversible tumorigenesis by MYC in hematopoietic lineages. *Mol. Cell* **4**, 199-207.
- Freytag, S. O.** (1988). Enforced expression of the c-myc oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G0/G1. *Mol. Cell Biol.* **8**, 1614-1624.
- Gandarillas, A. and Watt, F. M.** (1997). c-Myc promotes differentiation of human epidermal stem cells. *Genes Dev.* **11**, 2869-2882.
- Gardner, H. P., Belka, G. K., Wertheim, G. B., Hartman, J. L., Ha, S. I., Gimotty, P. A., Marquis, S. T. and Chodosh, L. A.** (2000). Developmental role of the SNF1-related kinase Hunk in pregnancy-induced changes in the mammary gland. *Development* **127**, 4493-4509.
- Gunther, E. J., Belka, G. K., Wertheim, G. B., Wang, J., Hartman, J. L., Boxer, R. B. and Chodosh, L. A.** (2002). A novel doxycycline-inducible system for the transgenic analysis of mammary gland biology. *FASEB J.* **16**, 283-292.
- Haggerty, T. J., Zeller, K. I., Osthus, R. C., Wonsey, D. R. and Dang, C. V.** (2003). A strategy for identifying transcription factor binding sites reveals two classes of genomic c-Myc target sites. *Proc. Natl. Acad. Sci. USA* **100**, 5313-5318.
- Heath, V. J., Gillespie, D. A. and Crouch, D. H.** (2000). Inhibition of the terminal stages of adipocyte differentiation by cMyc. *Exp. Cell Res.* **254**, 91-98.
- Helmrich, S. P., Shapiro, S., Rosenberg, L., Kaufman, D. W., Slone, D., Bain, C., Miettinen, O. S., Stolley, P. D., Rosenshein, N. B., Knapp, R. C. et al.** (1983). Risk factors for breast cancer. *Am. J. Epidemiol.* **117**, 35-45.
- Hennighausen, L. G. and Sippel, A. E.** (1982). Characterization and cloning of the mRNAs specific for the lactating mouse mammary gland. *Eur. J. Biochem.* **125**, 131-141.
- Hennighausen, L., Robinson, G. W., Wagner, K. U. and Liu, X.** (1997). Developing a mammary gland is a stat affair. *J. Mammary Gland Biol. Neoplasia* **2**, 365-372.
- Hildreth, N. G., Shore, R. E. and Dvoretzky, P. M.** (1989). The risk of breast cancer after irradiation of the thymus in infancy. *N. Engl. J. Med.* **321**, 1281-1284.
- Humphreys, R. C. and Hennighausen, L.** (1999). Signal transducer and activator of transcription 5a influences mammary epithelial cell survival and tumorigenesis. *Cell Growth Differ.* **10**, 685-694.
- Humphreys, R. C., Bierie, B., Zhao, L., Raz, R., Levy, D. and Hennighausen, L.** (2002). Deletion of Stat3 blocks mammary gland involution and extends functional competence of the secretory epithelium in the absence of lactogenic stimuli. *Endocrinology* **143**, 3641-3650.
- Hynes, N. E., Cella, N. and Wartmann, M.** (1997). Prolactin mediated intracellular signaling in mammary epithelial cells. *J. Mammary Gland Biol. and Neoplasia* **2**, 19-27.
- Iavnilovitch, E., Groner, B. and Barash, I.** (2002). Overexpression and forced activation of stat5 in mammary gland of transgenic mice promotes cellular proliferation, enhances differentiation, and delays postlactational apoptosis. *Mol. Cancer Res.* **1**, 32-47.
- Kelly, P. A., Bachelot, A., Kedzia, C., Hennighausen, L., Ormandy, C. J., Kopchick, J. J. and Binart, N.** (2002). The role of prolactin and growth hormone in mammary gland development. *Mol. Cell. Endocrinol.* **197**, 127-131.
- Kile, B. T. and Alexander, W. S.** (2001). The suppressors of cytokine signalling (SOCS). *Cell Mol. Life Sci.* **58**, 1627-1635.
- Land, C. E., Tokunaga, M., Koyama, K., Soda, M., Preston, D. L., Nishimori, I. and Tokuoka, S.** (2003). Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950-1990. *Radiat. Res.* **160**, 707-717.
- Layde, P. M., Webster, L. A., Baughman, A. L., Wingo, P. A., Rubin, G. L. and Ory, H. W.** (1989). The independent associations of parity, age at first full term pregnancy, and duration of breastfeeding with the risk of breast cancer. Cancer and Steroid Hormone Study Group. *J. Clin. Epidemiol.* **42**, 963-973.
- Lindeman, G. J., Wittlin, S., Lada, H., Naylor, M. J., Santamaria, M., Zhang, J. G., Starr, R., Hilton, D. J., Alexander, W. S., Ormandy, C. J. and Visvader, J.** (2001). SOCS1 deficiency results in accelerated mammary gland development and rescues lactation in prolactin receptor-deficient mice. *Genes & Dev.* **15**, 1631-1636.
- Liu, X., Robinson, G. W. and Hennighausen, L.** (1996). Activation of Stat5a and Stat5b by tyrosine phosphorylation is tightly linked to mammary gland differentiation. *Mol. Endocrinol.* **10**, 1496-1506.
- MacMahon, B., Cole, P., Lin, T. M., Lowe, C. R., Mirra, A. P., Ravnihar, B., Salber, E. J., Valaoras, V. G. and Yuasa, S.** (1970). Age at first birth and breast cancer risk. *Bull. World Health Organ.* **43**, 209-221.
- MacMahon, B., Purde, M., Cramer, D. and Hint, E.** (1982). Association of breast cancer risk with age at first and subsequent births: a study in the population of the Estonian Republic. *J. Natl. Cancer Inst.* **69**, 1035-1038.
- Marquis, S. T., Rajan, J. V., Wynshaw-Boris, A., Xu, J., Yin, G. Y., Abel, K. J., Weber, B. L. and Chodosh, L. A.** (1995). The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues. *Nat. Genet.* **11**, 17-26.
- Master, S. R., Hartman, J. L., D'Cruz, C. M., Moody, S. E., Keiper, E. A., Ha, S. I., Cox, J. D., Belka, G. K. and Chodosh, L. A.** (2002). Functional microarray analysis of mammary organogenesis reveals a developmental role in adaptive thermogenesis. *Mol. Endocrinol.* **16**, 1185-1203.
- Master, S. R., Stoddard, A. J., Bailey, L. C., Pan, T.-C., Dugan, K. D. and Chodosh, L. A.** (2005). Genomic analysis of early murine mammary gland development. *Genome Biol.* (in press).
- Miller, A. B., Howe, G. R., Sherman, G. J., Lindsay, J. P., Yaffe, M. J., Dinner, P. J., Risch, H. A. and Preston, D. L.** (1989). Mortality from breast cancer after irradiation during fluoroscopic examinations in patients being treated for tuberculosis. *New Engl. J. Med.* **321**, 1285-1289.
- Miner, J. H. and Wold, B. J.** (1991). c-myc inhibition of MyoD and myogenin-initiated myogenic differentiation. *Mol. Cell Biol.* **11**, 2842-2851.
- Miyoshi, K., Shillingford, J. M., Smith, G. H., Grimm, S. L., Wagner, K. U., Oka, T., Rosen, J. M., Robinson, G. W. and Hennighausen, L.** (2001). Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. *J. Cell Biol.* **155**, 531-542.
- Murphy, W., Sarid, J., Taub, R., Vasicek, T., Battey, J., Lenoir, G. and Leder, P.** (1986). A translocated human c-myc oncogene is altered in a conserved coding sequence. *Proc. Natl. Acad. Sci. USA* **83**, 2939-2943.
- Myal, Y., Robinson, D. B., Iwasiow, B., Tsuyuki, D., Wong, P. and Shiu, R. P.** (1991). The prolactin-inducible protein (PIP/GCDFP-15) gene: cloning, structure and regulation. *Mol. Cell. Endocrinol.* **80**, 165-175.
- Neville, M. C., McFadden, T. B. and Forsyth, I.** (2002). Hormonal regulation of mammary differentiation and milk secretion. *J. Mammary Gland Biol. Neoplasia* **7**, 49-66.
- Nguyen, A. V. and Pollard, J. W.** (2000). Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. *Development* **127**, 3107-3118.
- Park, D. S., Razani, B., Lasorella, A., Schreiber-Agus, N., Pestell, R. G., Iavarone, A. and Lisanti, M. P.** (2001). Evidence that Myc isoforms transcriptionally repress caveolin-1 gene expression via an INR-dependent mechanism. *Biochemistry* **40**, 3354-3362.
- Park, D. S., Lee, H., Frank, P. G., Razani, B., Nguyen, A. V., Parlow, A. F., Russell, R. G., Hulit, J., Pestell, R. G. and Lisanti, M. P.** (2002). Caveolin-1-deficient mice show accelerated mammary gland development

- during pregnancy, premature lactation, and hyperactivation of the Jak-2/STAT5a signaling cascade. *Mol. Biol. Cell* **13**, 3416-3430.
- Pelengaris, S., Littlewood, T. D., Khan, M., Elia, G. and Evan, G.** (1999). Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion. *Mol. Cell* **3**, 565-577.
- Pelengaris, S., Khan, M. and Evan, G. I.** (2002). Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. *Cell* **109**, 321-334.
- Quarrie, L. H., Addey, C. V. and Wilde, C. J.** (1996). Programmed cell death during mammary tissue involution induced by weaning, litter removal, and milk stasis. *J. Cell Physiol.* **168**, 559-569.
- Ren, S., Cai, H. R., Li, M. and Furth, P. A.** (2002). Loss of Stat5a delays mammary cancer progression in a mouse model. *Oncogene* **21**, 4335-4339.
- Russo, J., Rivera, R. and Russo, I. H.** (1992). Influence of age and parity on the development of the human breast. *Breast Cancer Res. Treat.* **23**, 211-218.
- Schoenenberger, C. A., Andres, A. C., Groner, B., van der Valk, M., LeMeur, M. and Gerlinger, P.** (1988). Targeted c-myc gene expression in mammary glands of transgenic mice induces mammary tumours with constitutive milk protein gene transcription. *EMBO J.* **7**, 169-175.
- Shore, R. E., Hempelmann, L. H., Kowaluk, E., Mansur, P. S., Pasternack, B. S., Albert, R. E. and Haughie, G. E.** (1977). Breast neoplasms in women treated with x-rays for acute postpartum mastitis. *J. Natl. Cancer Inst.* **59**, 813-822.
- Stampfer, M. R., Yaswen, P., Alhadeff, M. and Hosoda, J.** (1993). TGF beta induction of extracellular matrix associated proteins in normal and transformed human mammary epithelial cells in culture is independent of growth effects. *J. Cell Physiol.* **155**, 210-221.
- Stewart, T. A., Pattengale, P. K. and Leder, P.** (1984). Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. *Cell* **38**, 627-637.
- Teglund, S., McKay, C., Schuetz, E., van Deursen, J. M., Stravopodis, D., Wang, D., Brown, M., Bodner, S., Grosveld, G. and Ihle, J. N.** (1998). Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell* **93**, 841-850.
- Timme, T. L., Goltsov, A., Tahir, S., Li, L., Wang, J., Ren, C., Johnston, R. N. and Thompson, T. C.** (2000). Caveolin-1 is regulated by c-myc and suppresses c-myc-induced apoptosis. *Oncogene* **19**, 3256-3265.
- Watson, P. H., Safneck, J. R., Le, K., Dubik, D. and Shiu, R. P.** (1993). Relationship of c-myc amplification to progression of breast cancer from in situ to invasive tumor and lymph node metastasis. *J. Natl. Cancer Inst.* **85**, 902-907.