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Regulation of Apoptosis During Mammary Involution by the p53 Tumor Suppressor Gene

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ABSTRACT

Regulation and functions of the p53 tumor suppressor gene have been studied extensively with respect to its critical role in maintaining the stability of genomic DNA following genotoxic insults. However, p53 is also induced by physiologic stimuli resulting in cell cycle arrest and apoptosis. In other situations, the activity of p53 must be repressed to prevent inappropriate removal of cells. The mammary gland provides a valuable system in which to study the mechanisms by which the expression and biological responses to p53 can be regulated under a variety of physiological circumstances. The pro-apoptotic role of p53 in the secretory mammary epithelium may be especially relevant to lactation in livestock. We have utilized p53-deficient mice to establish the molecular targets of p53 in the mammary gland and biological consequences when it is absent. The p21/WAF1 gene (*Cdkn1a*) is a transcriptional target gene of the p53 protein that responds to elevated levels of p53 during milk stasis providing an endogenous reporter of p53 activity. Abrogation of p53 resulted in delayed involution of the mammary epithelium, demonstrating the physiological role of p53 in regulating involution. Though delayed, stromal proteases were induced in the mammary gland by 5 d postweaning, providing a p53-independent mechanism that resulted in removal of the residual secretory epithelium. These processes can be interrupted by treatment with hydrocortisone. These data establish p53 as a physiological regulator of involution that acts to rapidly initiate apoptosis in the secretory epithelium in response to stress signals, but also indicate the presence of compensatory pathways to effect involution. Additional mechanisms involving intracellular stress signaling pathways (e.g., Stat3) and stromal-mediated pathways have been identified and, together with p53 pathways, may be used to identify animals with greater persistency of lactation.

(**Key words:** p53, Stat3, involution, lactation)

Abbreviation key: TIMP = tissue inhibitors of metaloproteinases.

INTRODUCTION

Milk yield has been a major selection criterion for genetic improvement in livestock. In dairy animals, selection for increased milk yield has resulted in dramatic increases in production (Kelm and Freeman, 2000; Wiggans, 1991). Lactation performance is not selected for directly in meat animals, but remains a significant factor influencing neonatal growth rates and productivity (Irgang et al., 1985; Miller et al., 1999). Lactation is a growing concern in swine as litter size can exceed the ability of the dam to support optimal growth of neonates (Britt, 1986; Shurson and Irvin, 1992). Milk yield is determined by numerous factors that act systemically as well as factors acting directly within the mammary gland. Growth and differentiation of the glandular epithelium during puberty and pregnancy are important determinants of the total area of secretory epithelium and milk yield (Cowie et al., 1980; Sejrsen et al., 1982, 2000). The molecular mechanisms controlling proliferation and signaling pathways regulating milk protein gene expression have been examined in great detail (Robinson et al., 1996). Maintenance of the secretory epithelium is also a critical parameter determining persistency of lactation and overall milk yield (Knight and Peaker, 1984; Capuco and Akers, 1999). The mammary epithelial area declines by ~50% in ruminants during the declining phase of lactation (Wilde and Knight, 1989; Capuco et al., 2001), but the description of the molecular pathways has evolved only recently.

Integrity of the secretory epithelium during lactation depends on a balance of survival and death signals, which regulate programmed cell death. The realization that cell death pathways are disrupted in a majority of human tumors prompted intense study of the biochemical pathways involved in apoptosis. Elucidation of the critical players in apoptotic pathways prompted the investigation of how these pathways are regulated in nor-

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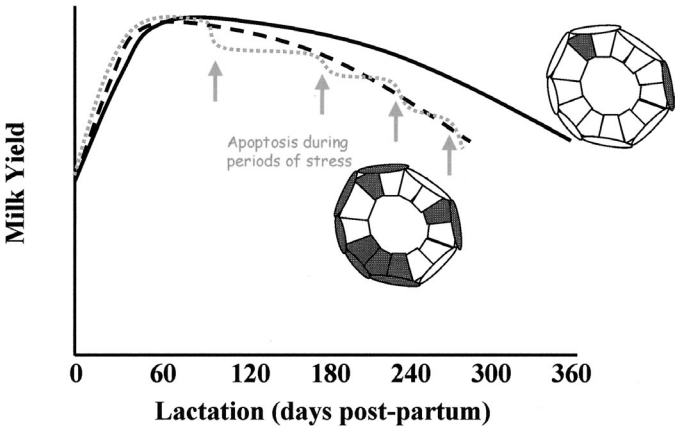

Figure 1. Hypothetical lactation curves for dairy cattle in which the solid line represents a typical lactation with an alveolus that is largely intact and functional. The dotted line demonstrates how periodic and localized interruptions in milk removal may lead to irreversible loss of secretory epithelium (shown in adjacent alveolus as stippled cells) and less persistent lactation represented by the dashed line.

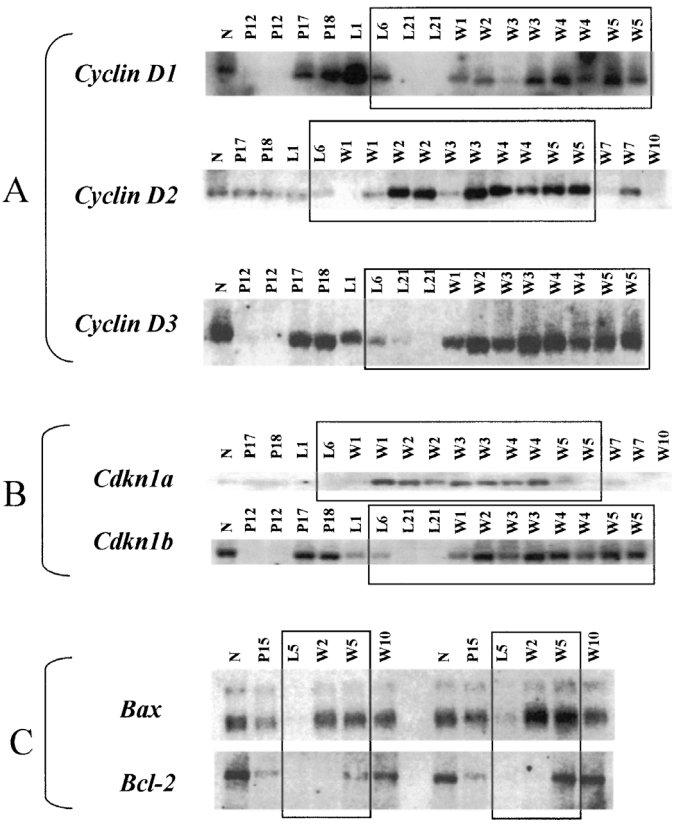

Figure 2. Patterns of gene expression during mammary gland development and involution in mice. Stages of mammary gland development include nulliparous (N); d 12, 15, 17, 18 of pregnancy (P12, P15, P17, P18), d 1, 5, 6, 21 of lactation (L1, L5, L6, L21), and days postweaning (W1, W2, W3, W4, W5, W10). Lanes within the boxes emphasize the changes in expression of the genes during transition from peak lactation (d 5 to 10) through the first phase of involution (d 1 to 5 postweaning). Genes associated with cell cycle progression (panel A), cell cycle inhibition (panel B), and apoptosis (panel C) show rapid induction following removal of pups during early lactation (5 d postpartum). All blots were reprobed with *gapdh* or 18S rRNA probes to assure equal loading (data not shown).

mal tissues as well. Programmed cell death is a fundamental mechanism required for proper development of embryos and organs in virtually all multicellular organisms. In adult animals, involution of the mammary epithelium following weaning of neonates provides a dramatic example of programmed cell death in which a substantial portion of the mammary epithelium is removed by apoptosis. In mice, > 80% of the epithelium is eliminated following forced weaning of pups soon after lactation is established (Walker et al., 1989). Signals induced by milk stasis must act locally to shift the balance of pro-apoptotic and anti-apoptotic factors within the mammary epithelium because involution can proceed in one gland without interrupting lactation in adjacent glands (Lund et al., 1996; Li et al., 1997). Therefore, localized disruptions in milk removal can result in a loss of secretory epithelium and a decline in milk yield throughout lactation (Figure 1).

Milk stasis causes distention of alveoli leading to mechanical stress as well as restricted blood flow and hypoxia. Local accumulation of feedback inhibitors of lactation have been proposed (Peaker and Wilde, 1996) as mediators as well. Although the signals that initiate involution remain to be identified, the expression of a number of transcription factors and stress-response genes is observed within hours after milk stasis (Strange et al., 1992; Jaggi et al., 1996). Although terminally differentiated, DNA synthesis is initiated within the mammary epithelial cells by 48 h following milk stasis (Wiesen and Werb, 2000). This is associated with the induction of "immediate-early genes" (e.g., myc, fos, jun) and cell cycle-associated genes (Figure 2A), but inhibitors of cell cycle are also increased (Figure 2B). Pro-apoptotic genes appear to be induced with nearly the same kinetics. Bax shows a rapid induction, while expression of the anti-apoptotic gene *Bcl-2* is not expressed until later (Figure 2C) when remodeling becomes prominent and apoptosis is diminished. A change in the ratio of Bcl-X_s/Bcl-X_L that would favor apoptosis is also observed during early involution (Heermeier et al., 1996). After the initial phase of apoptosis, expression of proteases (e.g., stromelysin-1) marks a secondary phase of involution that allows remodeling of the ductal architecture (Lund et al., 1996). Therefore, milk stasis initiates a dramatic upheaval in the patterns of gene expression and engages a broad range of biological

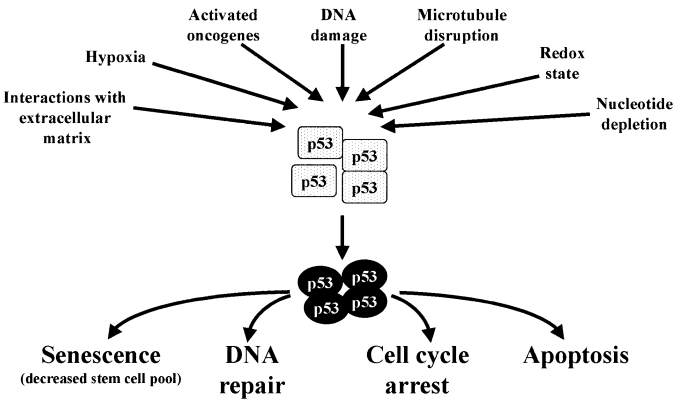

Figure 3. Pathways for activation of p53 responses. Multiple stimuli lead to posttranslational modification of p53 protein to form active tetramers that stabilize p53 protein and enhance its sequence-specific DNA binding leading to transcriptional activation of target genes. Depending on the cellular context and stimuli, activation of p53 may induce senescence, facilitate DNA repair, initiate transient cell cycle arrest or induce apoptosis.

responses. But these results alone cannot discern which genes are key regulators of involution.

THE ROLE OF P53 IN APOPTOSIS OF THE MAMMARY EPITHELIUM

The p53 tumor suppressor gene (designated *Trp53* in mouse; *TP53* in humans) responds to a wide array of physiological and genotoxic stresses (Figure 3) and has prompted detailed analysis of its functions. Although p53 protein is expressed constitutively in most cells, its levels remain very low due to its rapid degradation. The p53 protein is a substrate for a variety of enzymes induced by DNA damage or cell stress resulting in posttranslational modifications (phosphorylation, acetylation, ribosylation, SUMOylation). Posttranslational modifications in p53 promote the formation of tetramers and dissociation from factors that mediate its ubiquitin-dependent degradation (e.g., MDM2) or inhibit its transcriptional activation domain (e.g., MDMX). These changes lead to accumulation of p53 protein within the nucleus and augmentation of its sequence-specific DNA-binding activity and transcriptional activation of target genes. The *Cdkn1a* gene (encoding p21/WAF1 protein) is an important target gene that is induced following activation of p53. *Cdkn1a* encodes an inhibitor of cyclin-dependent kinases that inhibit phosphorylation of retinoblastoma protein resulting in cell cycle arrest at the G1/S boundary (El-Deiry et al., 1993). This would appear to be a prudent strategy to allow inspection and repair of the DNA to prevent cells from replicating damaged DNA. If, however, the genetic damage is severe, p53 also transcriptionally activates pro-apoptotic genes such as Bax and Fas, which is augmented by transrepression of anti-apoptotic genes such as Bcl-2 (Somasundaram, 2000). The pivotal role of p53 in sensing and orchestrating appropriate responses to genotoxic damage has led to it being dubbed "guardian of the genome" (Lane, 1992).

Physiological stresses such as hypoxia, depletion of nucleotides, redox status, loss of attachment to adjacent cells or substratum have also been shown to induce p53 activity. The heavy metabolic demands of lactation may lead to depletion of substrates or altered redox, causing activation of p53 as well as other stress-response pathways, which can initiate apoptosis. Indeed, expression of the *Trp53* gene is induced rapidly within the mammary epithelium following milk stasis in mice, suggesting that it may be a signal that initiates apoptosis (Strange et al., 1992). Removal of pups from lactating female mice resulted in a rapid induction of p53 mRNA and protein. The concentrations of p21/WAF1 mRNA were elevated more than eightfold but were absent in mice in which the wild type allele of p53 had been deleted (Jerry et al., 1998). Therefore, the *Trp53* tumor suppressor gene can be regulated by physiological stimuli and the *Cdkn1a* gene provides an endogenous reporter of p53 activity within mammary epithelial cells.

GENETICALLY ENGINEERED MICE FOR THE STUDY OF INVOLUTION

Genetically engineered mice provide a powerful tool to identify pathways that participate in involution. Specific pathways can be disrupted by overexpression of dominant-negative forms of proteins, expression of antisense mRNA as well as targeted mutations introduced by homologous recombination in embryonic stem cells to determine the effects on complex biological processes. These approaches have been applied to begin identifying genes that are essential for involution.

Transgenic mice overexpressing various genes that may be involved in involution provide one means to identify genes that initiate involution and establish cause-and-effect relationships. Expression of the immediate-early response genes (e.g., myc) cause disruptions in proliferation and tumor formation rather than premature involution (Amundadottir et al., 1995), suggesting that these genes are not effectors of involution. Delays in involution have been described in transgenic mice overexpressing Bcl-2, IGF-I, and IGF-binding proteins along with increased incidence of mammary tumors (Hadsell et al., 1996; Jager et al., 1997; LeRoith et al., 1995; Neuenschwander et al., 1996). These experiments are often complicated because inappropriate activation of proliferation often results in induction of p19ARF, which can initiate p53-dependent apoptosis

(de Stanchina et al., 1998; Lowe et al., 1994). Therefore, activation of oncogenic pathways often results in premature apoptosis of the mammary epithelium (Li et al., 1996; Lundgren et al., 1997; Tzeng et al., 1998) or neoplastic changes that alter the gland so dramatically that the role of the transgene in involution is difficult to assess. In contrast, transgenic mice expressing high levels of a mutant form of p53 resulted in premature apoptosis and failure of lactation (Li et al., 1994). Although unexpected, this particular mutant of p53 retained activities of the wild type p53 protein inducing apoptosis in the mammary gland as well as resistance to tumor formation (Li et al., 1995).

Analysis of mammary glands from mice that were homozygous for a null allele of the p53 tumor suppressor gene (*Trp53*$^{-/-}$) revealed no defects in growth or differentiation of the tissue. However, alveoli distended with milk were detected in histological sections from *Trp53*$^{-/-}$ females even at 2 wk postweaning (Jerry et al., 1999). A detailed analysis of mammary glands from p53-deficient mice revealed a delay in the rate of involution (Jerry et al., 1998). The effect was transient as differences in epithelial area between *Trp53*$^{+/+}$ and *Trp53*$^{-/-}$ were evident at 2 to 5 d postweaning but were indistinguishable by 7 d postweaning. Although apoptosis of the mammary epithelium was delayed in *Trp53*$^{-/-}$ females, transcription of milk protein genes was extinguished within 48 h postweaning in both wild type and *Trp53*$^{-/-}$ females. Similarly, induction of stromal proteases proceeded on cue at 5 d postweaning regardless of whether *Trp53* was present or absent (Jerry et al., 1998). The stromal proteases appeared to initiate a secondary wave of apoptosis in response to chronic stress signals, which proceed by p53-independent pathways allowing involution to recover by 7 d postweaning in *Trp53*$^{-/-}$ females. Therefore, p53 regulates only the initial wave of apoptosis in response to acute stress, but does not affect milk protein gene expression or apoptotic responses to chronic stress.

Transcriptional activation of the *Trp53* gene is one mechanism by which the p53 pathway is activated during transition from lactation to involution. However, p53 activity varies dramatically among stages of mammary gland development and differentiation (Kuperwasser et al., 2000), suggesting additional levels of control. Given the dire consequence of apoptotic cell death if p53 should be inappropriately activated, it is not surprising that antagonists are also present to enforce strict regulation of p53. The MDM2 protein would appear to be a potent inhibitor of p53 as it acts at multiple levels (Momand et al., 2000). First, MDM2 binds to the N-terminus of p53 to directly inhibit its transactivation domain. Second, it mediates the active export of p53 from the nucleus to the cytoplasm. Lastly, MDM2 ubiquinates p53 protein targeting it for rapid degradation by the proteasome. Though MDM2 was shown to be expressed in the mammary gland (Pinkas et al., 1999), there was no clear association with p53 activity. Furthermore, alternative transcripts unique to the mammary gland were detected in both mouse and human. The biochemical functions of the proteins encoded by these mammary-specific variants of MDM2 remain unclear. Stress signals engage additional players in the p53 pathway. The induction of DNA synthesis during involution may be recognized as illegitimate proliferation in terminally differentiated mammary epithelial cells. The p19/ARF protein (alternative reading frame of the *INK4A* gene encoding the p15 protein in human and p19 protein in mouse) is induced in response to inappropriate mitogenic stimulation in normal cells and mediates p53-dependent apoptosis (de Stanchina et al., 1998). The p19/ARF protein forms a ternary complex with MDM2 and p53, which inhibits the MDM2-mediated ubiquitination and degradation of p53 (Kamijo et al., 1998). The p19/ARF protein was also shown to vary among stages of mammary gland development. A spike in p19/ARF was observed only at 2 d postweaning, which coincided with the maximal activation of p53-dependent expression of p21/WAF1. Therefore, alterations in the balance between MDM2 and p19/ARF appears to regulate apoptosis within the mammary epithelium in response to milk stasis (Figure 4).

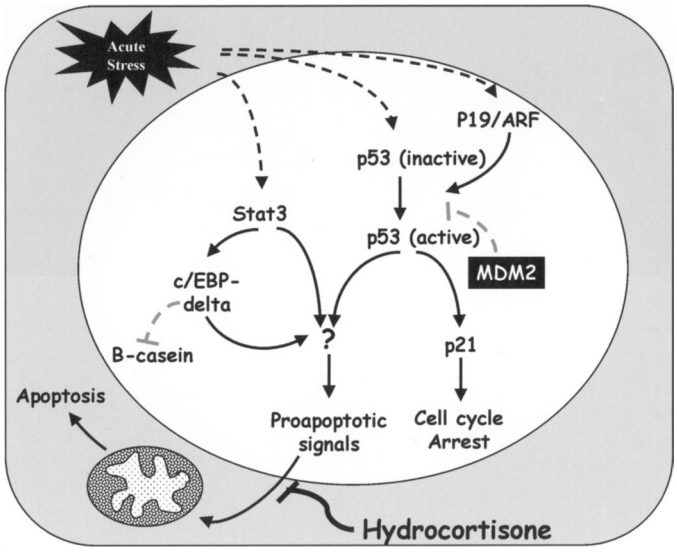

Figure 4. Pathways influencing involution of the mammary epithelium. Physiologic stress of milk stasis has been shown to engage p53- and Stat3-mediated pathways which both initiate apoptosis of mammary epithelial cells. Stat3 inhibits milk protein gene expression, while p53 enforces a block cell cycle. The activity of p53 is regulated by a dynamic balance in levels of activators (e.g., P19/ARF) and antagonists (e.g., MDM2).

Based on the many genes activated during involution and the pleotropic effects of cellular stress, other pathways would also be expected to participate in involution. Expression of the Stat3 and IRF-1 genes was also induced in the mammary gland after weaning of neonates. Targeted disruption of Stat3 resulted in a transient delay in removal of the mammary epithelium following weaning (Chapman et al., 1999) that was strikingly similar to that observed in p53-deficient mice. Stat3 is a transcriptional activator that can regulate numerous genes including c/EBP-delta, which appears to play a role in apoptosis. Expression of c/EBP-delta inhibited transcription of the beta-casein gene (Hutt et al., 2000; O'Rourke et al., 1999). Although the mammary phenotype in Stat3-deficient mice resembles that of the p53-deficient mice, these appear to represent distinct pathways (Figure 4). First, expression of milk protein genes was prolonged in Stat3-deficient, but not p53-deficient mice suggesting that Stat3 may be a physiologic repressor of β-casein and possibly other milk protein genes. Second, levels of p53 and p21/WAF1 protein were increased in Stat-3-deficient mice and appear to participate in the compensatory involution. In contrast to the pro-apoptotic roles of the p53 and Stat3 pathways, targeted deletion of IRF-1 increased rates of involution (Chapman et al., 2000). These data demonstrate that IRF-1 represents another distinct pathway participating in acute responses to milk stasis in the mammary gland and serves to inhibit the loss of mammary epithelial cells.

As the matrix metaloproteinases (e.g., stromelysin-1) appear to participate in the process of involution, the balance of proteinases and tissue inhibitors of metaloproteinases (**TIMP**) represents another pathway that bears consideration. Expression of TIMP appears to be regulated by endocrine status (Fata et al., 2001a). Recently, involution of the mammary epithelium was shown to be accelerated in mice bearing homozygous deletions of the *Timp-3* gene (Fata et al., 2001b). Administration of a proteinase inhibitor (inhibits MMP as well as a disintegrin and metaloproteinase/"ADAM") restored the normal kinetics of involution, but was unable to prevent the process.

MANIPULATING INVOLUTION

Experiments in mice have identified pathways that regulate involution in a biphasic manner (Lund et al., 1996; Li et al., 1997; Jerry et al., 1998). It is clear that multiple pathways are present with overlapping and compensatory activities. During the initial phase of involution, the secretory epithelial cells sense localized stress resulting from the accumulation of milk within the lumen of alveoli. Activation of p53 and Stat3, along with repression of IRF-1 and TIMP-3, participate in this phase during which mammary epithelial cells commit mass suicide. Although the kinetics of involution was altered as a result of targeted disruption of these genes, it was equally clear that mechanisms regulating the second phase of involution were unperturbed. Phase 2 of involution involves the secretion of proteases by stromal cells in response to chronic stress cues. Normally, the stromal proteases initiate remodeling of the mammary epithelium and encourage the reemergence of the adipocytes (Thomasset et al., 1998; Alexander et al., 2001) that dominate the stroma of the nonpregnant mammary gland. However, premature expression of stromal proteases can disrupt the basement membrane and cell-cell adhesions involving integrins and cadherins. The loss of attachment of mammary epithelial cells results in apoptosis by pathways that appear to be independent of p53 or Stat3. Tissue inhibitors of metaloproteinases are natural antagonists of stromal proteases that are also expressed in the mammary epithelium during involution (Lund et al., 1996). As such, they provide a means for modifying the second phase of involution. Although these pathways can modulate rates of apoptosis and loss of epithelial cells, treatment with glucocorticoids, and to a lesser extent progesterone, has been shown to be an effective means to completely interrupt involution signals in rodents (Feng et al., 1995; Jaggi et al., 1996; Lund et al., 1996; Li et al., 1997). Although treatment with hydrocortisone inhibits involution, induction of p53 and p21/WAF1 were intact (Figure 5). Therefore, glucocorticoids appear to act downstream of p53 to inhibit apoptosis and tissue remodeling.

The relative contributions of each of the intracellular signaling pathways and stromal-mediated pathways have not been investigated in livestock. The importance of these pathways may differ greatly between rodent models and livestock. Experimental intervention in rodents is usually conducted at peak lactation in an effort to synchronize the signals initiating involution to produce a more homogeneous response throughout the epithelium. In contrast, the dry period in dairy animals occurs in the declining phase of lactation. Rather than a synchronous loss of epithelium, the dry period is associated with the loss of expression of milk protein genes (Wiens et al., 1992; Schanbacher et al., 1993). Furthermore, dairy cattle are usually pregnant during the dry period, which may mask or balance the apoptotic signals. Therefore, in the practical management setting, involution in dairy cattle is confounded with mammogenic signals in preparation for the next lactation. Expression of stromal proteases may be relieved by the effects of increased progesterone or glucocorticoids (Feng et al., 1995; Jaggi et al., 1996). This feature of

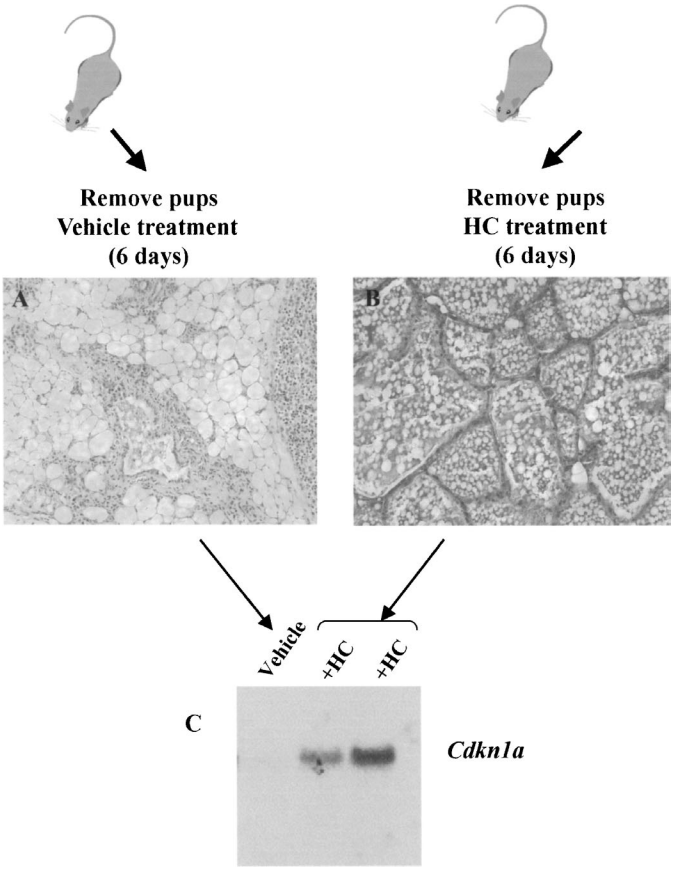

Figure 5. Effects of hydrocortisone treatment on involution and expression of *Cdkn1a*. Female mice were force-weaned at peak lactation (d 5 to 10 of lactation), then treated with either saline (Vehicle or hydrocortisone (HC treatment) for 6 d. Histological sections of mammary glands were stained with hematoxylin and eosin, and tissue was harvested for isolation of total RNA. **(A)** Milk stasis induced by forced-weaning results in rapid involution of the mammary epithelium in mice receiving Vehicle treatment, which is characterized by infiltration of unilocular adipocytes, reduction in epithelium present, absence of lobuloalveolar structures. **(B)** In contrast, treatment with hydrocortisone (HC treatment) blocks these processes and allows the maintenance of lobuloalveolar structures containing secretory products. Milk stasis for 6 d causes a rapid induction of p53. Transient induction of *Cdkn1a* is observed during d 1 to 5 postweaning (as shown in Figure 2B), which coincides with the period of maximal apoptosis in the mammary epithelium. **(C)** In Vehicle-treated mice, involution is largely complete by 6 d postweaning, resulting in a decline in *Cdkn1a* to basal levels. In contrast, integrity of the mammary epithelium is maintained in HC treated females in spite of sustained elevation of p53 and *Cdkn1a* mRNA. Therefore, hydrocortisone acts downstream of p53 to block involution.

management practices may account partially for differences in the rate of involution between rodents and livestock.

Dairy animals may be expected to have altered responses to milk stasis due to selection for animals that are resistant to the acute stress signals that can lead to cessation of lactation. As persistency of lactation appears to be a heritable trait, identification of polymorphisms in specific genes that segregate with variations in persistency of lactation in livestock will provide evidence of the practical importance of regulating involution and will allow direct selection for greater persistency. Conversely, rapid cessation of lactation and involution of mammary epithelial cells are important to minimize the risk of mastitis during the dry period. In this case, efforts to augment pro-apoptotic signals would be required. Compounds that stabilize the activity of pro-apoptotic pathways in the mammary epithelial cells could be administered to increase the efficiency and speed of involution. Activity of proteases secreted by the stromal cells may also be manipulated to facilitate phase 2 of involution. As the first and second phases of involution appear to be regulated by distinct pathways and are separated temporally (Lund et al., 1996; Li et al., 1997; Jerry et al., 1998), it may be possible to identify polymorphisms in genes that minimize apoptotic responses to acute disruptions in milk removal (inhibit phase 1 of involution) while preserving polymorphisms in genes that assure faithful removal of the mammary epithelium in response to chronic disruption of milk removal (activators of phase 2 of involution). In this way, losses of mammary epithelium during lactation could be prevented while assuring rapid involution during the dry period.

SUMMARY

Mouse models have provided direct evidence identifying distinct pathways that participate in responses to acute and chronic disruptions in milk removal and cause apoptosis of the secretory cells of the mammary gland. Acute responses involve activation of p53 and Stat3 and repression of IRF-1. Chronic stress invokes release of stromal proteases, which can be countered by expression of TIMP. Therefore, a complex network of redundant mechanisms assures involution of the mammary epithelium. The present challenge will be to identify pathways that are relevant to maintenance of lactation in livestock and develop strategies to manipulate these pathways for the purposes of improving lactation performance. The complexity of the mechanisms offers a rich source of targets that can be modified through transgenic approaches, endocrine manipulations as well as exploiting natural polymorphisms for genetic selection.

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