

Genetic Manipulation of the IGF-I Axis to Regulate Mammary Gland Development and Function

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ABSTRACT

Insulin-like growth factor I (IGF-I) is known to regulate mammary gland development. This regulation occurs through effects on both cell cycle progression and apoptosis. Our laboratory has studied the IGF-I-dependent regulation of these processes by using transgenic and knockout mouse models that exhibit alterations in the IGF-I axis. Our studies of transgenic mice that overexpress IGF-I during pregnancy and lactation have demonstrated that this growth factor slows the apoptotic loss of mammary epithelial cells during the declining phase of lactation but has minimal effects during early lactation on milk composition or lactational capacity. In contrast, our analysis of early developmental processes in mammary tissue from mice carrying a targeted mutation in the IGF-I receptor gene suggests that IGF-dependent stimulation of cell cycle progression is more important to early mammary gland development than potential anti-apoptotic effects. With both models, the effects of perturbing the IGF-I axis are dependent on the physiological state of the animal. The diminished ductal development that occurs in response to loss of the IGF-I receptor is dramatically restored during pregnancy, whereas the ability of overexpressed IGF-I to protect mammary cells from apoptosis does not occur if the mammary gland is induced to undergo forced involution. Data from our laboratory on the expression of IGF-signaling molecules in the mammary gland suggest that this effect of physiological context may be related to the expression of members of the insulin receptor substrate family.

(Key words: IGF-I, mammary, transgenic, knockout)

Abbreviation key: E2 = estradiol, GH = growth hormone, IGF = insulin-like growth factor, IGF-IR = IGF-I receptor, *Igflr* = IGF-IR gene, IGFBP = IGF binding

protein, IR = insulin receptor, IR-A and IR-B = insulin receptor isoforms A and B, respectively, IRS = insulin receptor substrate, TEB = terminal end bud, WAP-des = whey acidic protein-des(1-3)hIGF-I.

INTRODUCTION

Members of the insulin-like growth factor (IGF) family have been shown to act as central regulators of mammalian growth and metabolism, and IGF-I has long been thought to specifically mediate the actions of growth hormone (GH). Much of our current understanding of IGF-I action in animals has resulted from the application of transgenic and knockout mouse models. The development of these models has provided incredibly powerful tools for understanding both normal biology and the nature of abnormalities that occur during disease processes. In some cases, these models have provided ultimate proof of principle for hypotheses based on results of other experimental systems; in other cases, they have produced surprises that lead to reevaluation of existing thought. In addition, these models have served as a fertile testing ground for the development of new biotechnologies of commercial interest. The research field of mammary gland biology was among the earliest to use and benefit from these genetic technologies. The mouse mammary gland continues to be a very useful system for the application of transgenic and knockout technologies because of the availability of functional promoter DNA from mammary epithelium-specific genes, ease of accessibility for sampling purposes, and the ability to accurately recapitulate normal developmental programs after grafting.

The normal development of the mammary gland depends on the orchestrated interactions of a wide array of signaling pathways in response to endocrine signals and tissue-specific spatial queues. The importance of GH, prolactin, estrogen, and progesterone to normal mammary gland development and function has been known for almost half a century (Nandi, 1958). Yet, the mechanisms by which some of these hormones act have only recently been determined. The establishment of

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these mechanisms has emanated, in part, from studies conducted in transgenic or knockout mouse models. The models used in these studies have generally taken one of the following five distinct forms: 1) tissue-specific overexpression for gain of function, 2) tissue-specific overexpression of a dominant negative, 3) whole animal gene knockout, 4) tissue-specific conditional knockout, or 5) grafted knockout tissue in a normal host animal. These models have been useful in establishing the importance of local growth factor production as a mechanism for mediating hormone action on the mammary gland. Data from a variety of sources support the idea that growth factors produced within the mammary gland play an important role as local regulators of developmental processes, cellular differentiation, and cellular metabolism. The objective of this review was to highlight the contribution that genetically engineered mouse models have made to the understanding of IGF action on the mammary gland.

THE IGF AXIS: INTERORGAN TO INTRACELLULAR

The IGF Family

The IGF family is made up of the following three related ligands: insulin, IGF-I, and IGF-II. Both IGF-I and II are found in circulation and extracellular fluids conjugated to any one of six different IGF binding proteins (**IGFBP**) (Clemmons, 1999). As many as five different classes of membrane receptors may interact with ligands in the IGF family. This family of receptors consists of two splice variants of the insulin receptor (**IR-A** and **IR-B**), the type I IGF receptor (**IGF-IR**), and two potential hybrid receptors based on the dimerization of IGF-IR with either of the two insulin receptor (**IR**) splice variants (Bailly et al., 1997; Frasca et al., 1999). The biological actions of IGF-I, IGF-II, and insulin are currently believed to be mediated through IGF-IR, IR-A and IR-B, respectively (Morrione et al., 1997; Sciacca et al., 1999). A sixth receptor, the IGF-2/mannose-6-phosphate receptor, is a nonsignaling receptor that is believed to play a major role in the clearance of IGF-II from the circulation (Lau et al., 1994). The biological significance of the hybrid receptors is currently unclear. The predominant physiological action of IGF-I is the stimulation of postnatal body growth. This action was clearly demonstrated by measuring the change in BW over time in mice that carried targeted mutations of the genes for IGF-I and IGF-II (DeChiara et al., 1990; Liu et al., 1993). In addition, IGF-I can regulate aspects of whole-body protein synthesis, glucose uptake by peripheral tissues, and lipid metabolism (Fryberg, 1994; Sjogren et al., 2001; Yakar et al., 2001). The most frequently studied aspect of IGF-I action has been cell proliferation. In this respect, IGF-I has been reported

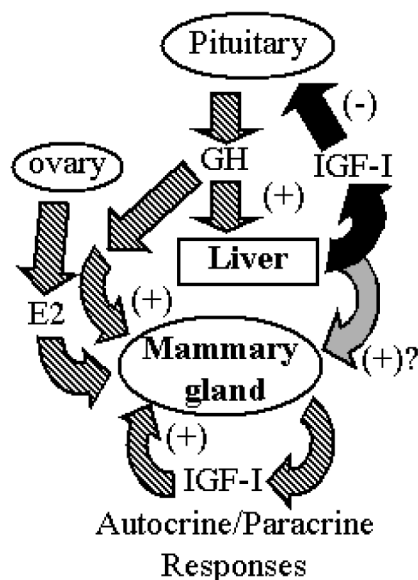


Figure 1. Application of the growth hormone/insulin-like growth factor I (GH/IGF-I) axis to the virgin mammary gland. Interactions among estrogen (E2), GH, and IGF-I are diagrammed in relationship to the mammary gland. All three have direct stimulatory effects (striped arrow) on mammary development. In addition, IGF-I produced by the liver in response to GH has negative feedback effects (black arrow) on GH secretion by the pituitary. The stimulation of mammary gland development by IGF-I occurs through local paracrine effects and potential endocrine-type effects (shaded arrow).

to be necessary for progression of cells through both the G1 and the G2/M phases of the cell cycle (Adesanya et al., 1999; Stiles et al., 1979). Another important aspect of IGF-I that has received considerable attention recently is the ability to protect cells from various forms of death. Cell death has been demonstrated to occur by the following three different pathways: apoptosis, anoikis, and necrosis (Coucouvani et al., 1995; Valentinis et al., 1998). Several cell types, including mammary cells, can be protected from both apoptosis and anoikis by the presence of IGF-I. The intracellular signaling pathways that mediate IGF-I-dependent cell survival have been extensively studied and are well defined. Other actions of IGF-I are highly cell type-specific and are outside the scope of this review (for a recent review, see LeRoith et al., 2001).

Origins and Evolution

The current understanding of IGF-I action places it at the bottom of an axis that involves GH (Figure 1). The axis was originally suggested almost half a century ago by Salmon and Daughaday (1957) as the somatomedin hypothesis. This hypothesis posited that the ability of GH to stimulate bone growth required the presence of a mediating factor, which later became

known as IGF-I (Rinderknecht and Humbel, 1978). In the years since its proposal, the somatomedin hypothesis has been tested in several other organ systems, including the mammary gland (Flint and Knight, 1997; Kleinberg, 1997). In addition, significant modifications have been made to the original hypothesis based on several more recent results, including those from the analysis of mice carrying a conditional liver-specific deletion of the IGF-I gene (LeRoith et al., 2001). The current form of the axis posits that GH released from the pituitary gland stimulates expression of IGF-I within both the liver and peripheral tissues (Figure 1). The presence of locally acting IGF-I within peripheral tissues then could cooperate with pituitary-derived GH to stimulate a biological response. This cooperativity was first described for adipocytes and has been referred to as the dual effector theory (Green et al., 1985). Discussion of the data for the dual effector theory can also be found in several reviews (Isaksson et al., 1987; Leroith et al., 2001; Ohlsson et al., 1998). Liver-derived IGF-I serves as an endocrine feedback regulator of GH secretion, and may also stimulate peripheral tissues, although this latter effect may be context-specific and is currently the subject of controversy (LeRoith et al., 2001; Lupu et al., 2001; Sjogren et al., 2001).

The action of IGF-I in the mammary gland has features similar to those described for other organ systems. However, some important distinctions exist. First, the analysis of GH and IGF-I action in the rodent and bovine mammary gland has demonstrated an important requirement for estrogen (estradiol, **E2**) within the IGF-I axis (Akers et al., 2000; Ruan et al., 1995). Second, the only evidence in support of a classical somatomedin-type action for IGF-I was found in studies of puberty-dependent mammary ductal development (Kleinberg et al., 1990; Weber et al., 2000). In fact, analysis of GH and IGF-I action during lactation has failed to provide consistent support for a somatomedin-type action of IGF-I on milk synthesis. In this respect, several important points must be made. First, accumulating data from both mouse and bovine models now supports that GH may be capable of directly stimulating milk synthesis during lactation (Gallego et al., 2001; Sinowatz et al., 2000; Yang et al., 2000). Second, although GH and E2 can increase IGF-I gene expression in the virgin mammary gland, a clear demonstration of GH-dependent induction of IGF-I gene expression in lactating mammary tissue has yet to be obtained (Glimm et al., 1992). Finally, IGF-I alone is insufficient to maintain milk secretion or mammary cell survival in the mammary glands of prolactin/GH-deficient rats (Flint et al., 1994; Travers et al., 1996). Although these observations do not prove that IGF-I is superfluous to the needs of lactation, they do support the idea that the nature and

regulation of the IGF-I axis in the lactating mammary gland may differ radically from those observed in the virgin mammary gland or in other organ systems.

Stated another way, the data on IGF-I action in the mammary gland illustrates the point that action of the IGF axis is context-specific. This has also been observed in the recent analysis of the effects of a CRE-recombinase mediated deletion of the IGF-I gene on whole-body growth versus glucose and lipid metabolism in mice (Sjogren et al., 1999, 2001). A major challenge in understanding IGF-I action lies in determining the basis for such specificity. Although context specificity could arise from a variety of factors, data from cell culture models suggest that the expression of intracellular IGF-I-dependent signaling molecules plays a dominant role in determining the nature of the IGF-I response (Baserga, 2000).

IGF Signaling Pathways

The activation of the IGF-IR is now known to generate intracellular signals through at least four distinct, but interacting, signal transduction pathways (Figure 2). The first pathway is a cell survival pathway that is activated through tyrosine phosphorylation of the scaffolding protein insulin receptor substrate 1 (**IRS-1**) (Nolan et al., 1997). The IRS-1 protein mediates IGF-I-dependent cell survival through activation of phosphatidylinositol 3'-kinase and the serine threonine kinase Akt (Datta et al., 1997). Overexpression of activated Akt within the mammary glands of transgenic mice causes delayed involution (Hutchinson et al., 2001; Schwertfeger et al., 2001). The second pathway is a proliferative pathway that involves activation of ras, raf, and erks 1 and 2 (Pelicci et al., 1992; Rozakis-Adcock et al., 1992; Sasoka et al., 1994). The activation of this pathway is known to stimulate cell cycle progression through coordinated regulation of cyclin D1 and p27^{kip1} (Aktas et al., 1997). Overexpression of cyclin D1 within the mammary glands of transgenic mice causes hyperplasias and tumors (Wang et al., 1994). The third pathway is a proliferative pathway that involves phosphorylation of β -catenin, which in turn is capable of stimulating expression of cyclin D1 and c-myc (Conover and Bale, 1998; Imbert et al., 2001; Playford et al., 2000). Overexpression of activated β -catenin or c-myc within the mammary glands of transgenic mice causes hyperplasias and tumors (Imbert et al., 2001; Stewart et al., 1984). The fourth pathway is a cell survival pathway that is mediated through an interaction of the IGF-IR with the 14.3.3 family of proteins (Craparo et al., 1997; Furlanetto et al., 1997). This interaction stimulates the translocation of the serine threonine kinase, raf1, to the mitochondria, resulting in the subsequent

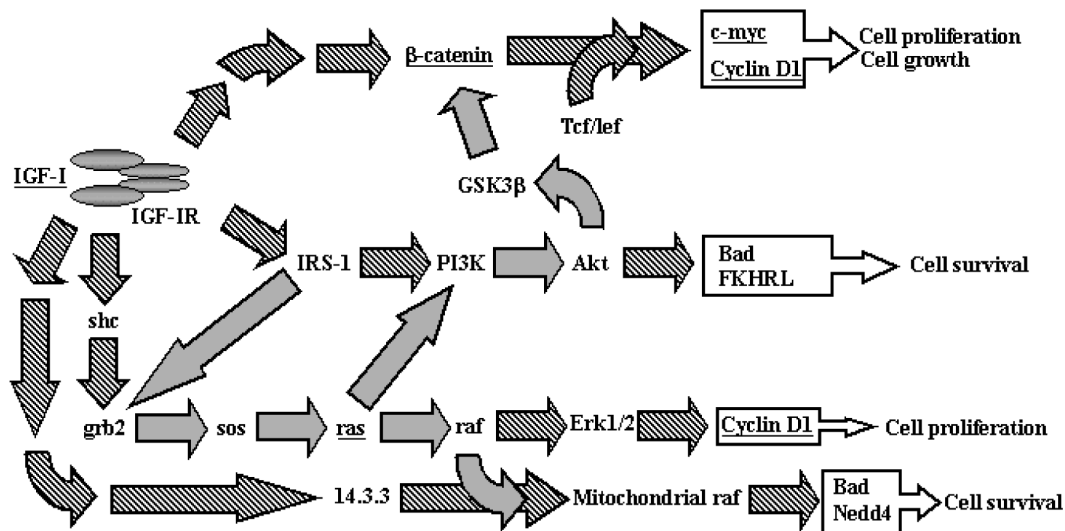


Figure 2. Insulin-like growth factor I receptor (IGF-IR)-dependent signal transduction pathways. The elements of this diagram represent functional links in four main signaling pathways (striped arrow) that have been worked out primarily through studies on cell culture models. Interactions between the pathways (shaded arrow) are also illustrated. Boxed arrows indicate mediators that are directly associated with the ultimate biological response. Underlined text indicates proteins that have been shown to cause mammary tumors in transgenic mice. GSK3 β = Glycogen synthase kinase 3 β , IRS-1 = insulin receptor substrate 1, PI3K = phosphatidylinositol 3'-kinase.

phosphorylation and inactivation of Bad (Peruzzi et al., 1999).

The presentation of these pathways as linear signal transmission events is done only for simplicity. Clear evidence exists, however, in support of cross-talk among the different pathways (Diehl et al., 1998; Rodriguez-Viciana et al., 1994; Skolnik et al., 1993). The existence of cross-talk among these different signaling pathways probably serves to provide additional levels of regulation in the form of redundancy or regulated amplification. Second, these pathways are not necessarily specific to the IGF-IR but can also be activated by several growth factor receptors. The activation of specific pathways by IGF-IR versus the other growth factor receptors is likely dependent on the cellular or physiological context. In this respect, much of the data supporting the signaling pathways described above comes from cell models. Thus, although the overexpression of several of the signaling proteins described above in transgenic mice can inhibit apoptosis or cause mammary hyperplasia or cancer, much remains to be learned about how these IGF-IR-dependent signaling molecules interact within the intact animal to drive normal mammary gland development.

IGF-I DRIVES VIRGIN MAMMARY DUCTAL DEVELOPMENT

The Terminal End Bud

In the mouse, the allometric stage of mammary gland development occurs with the onset of puberty and is

typified by the progressive infiltration of the mammary fat pad by a highly organized network of branching ducts. This ductal infiltration occurs through the proliferation of epithelial cells within specific structures called terminal end buds (TEB). At the leading edge of the TEB are two main populations of proliferative cells, cap cells and body cells. The cap cells and body cells give rise to myoepithelial and luminal epithelial cells, which can be distinguished by expression of α -smooth muscle actin and e-cadherin, respectively. A short distance behind this proliferative zone is a zone of apoptosis. This zone is responsible for canalization of the forming duct (Humphreys et al., 1996). Still further behind the zone of proliferation, the TEB narrows as a thickened basal lamina is deposited on the neck region. As the TEB penetrate the fat pad, branches are formed, either through bifurcation of existing TEB or through elongation of ducts that bud off the sides of more mature ducts.

IGF-I Acts on the TEB

A role of IGF-I in mammary ductal development has been inferred from several pieces of evidence. First, IGF-I can stimulate the proliferation of mammary epithelial cells in organ culture at low concentrations (Richert and Wood, 1999). Second, IGF-I, IGF-II, and the IGF-IR are expressed within both the epithelial and stromal compartments of the virgin mammary gland (Berry et al., 2001; Hovey et al., 1998; Richert and Wood, 1999; Yee et al., 1989). The expression of IGF-

I within the stromal compartment, coupled with the observation that both IGF-I and the IGF-IR are expressed within TEB supports the idea that IGF-I acts as a paracrine signal originating from the stroma and from select populations of cells within the TEB (Richert and Wood, 1999). Third, expression of IGF-I in mammary tissue can be elevated by exogenous GH and E2 (Kleinberg et al., 1990). Last, localized administration of IGF-I to the mammary gland of E2-treated hypophysectomized-ovariectomized rats can stimulate ductal development (Ruan et al., 1992). This increased ductal development is the result of increased TEB development. Although these observations supported a role for IGF-I in mammary ductal development, direct demonstration of the necessity of the IGF-I axis in this process was obtained through the analysis of mice with targeted mutations in the genes for IGF-I and the IGF-IR (Hadsell and Bonnette, 2000; Ruan and Kleinberg, 1999).

Targeted Mutations in the IGF System

Mice that carry a targeted mutation in the IGF-I gene have several phenotypic abnormalities, including reduced viability, dramatically reduced postnatal growth, and reduced fertility (Baker et al., 1996, 1997; Liu et al., 1993). In addition, the development of the mammary ductal system in the IGF-I knockout mice is tremendously impaired (Ruan and Kleinberg, 1999). The impaired ductal development was associated with diminished TEB development and could be restored only by the administration of exogenous E2 and IGF-I.

The need for the IGF-IR in mediating the actions of IGF-I on mammary gland development was demonstrated by the analysis of mammary gland grafts obtained from mice carrying a targeted mutation in the IGF-IR gene (*Igflr*^{-/-}) (Hadsell and Bonnette, 2000). Mutation of the IGF-IR in mice impairs fetal growth and is perinatally lethal. This lethality is caused by respiratory failure as a result of the impaired development of the intercostal muscles. Because of this perinatal lethality, the developmental potential of *Igflr*^{-/-} mammary tissue had to be analyzed by using mammary tissue transplantation techniques. The transplantation of fetal mammary tissue has become a powerful technique in the analysis of mammary gland development in mouse mutants. The application of this technique to the *Igflr*^{-/-} mice demonstrated that virgin mammary ductal development was dramatically impaired by loss of the IGF-IR (Hadsell and Bonnette, 2000). Mammary gland grafts from *Igflr*^{-/-} mice (Figure 3) also displayed dramatic reductions in both TEB number and size in comparison with *Igflr*^{+/+} mice (Bonnette and Hadsell, 2001). This developmental defect was associated with decreased numbers of 5-bromodeoxyuridine-labeled

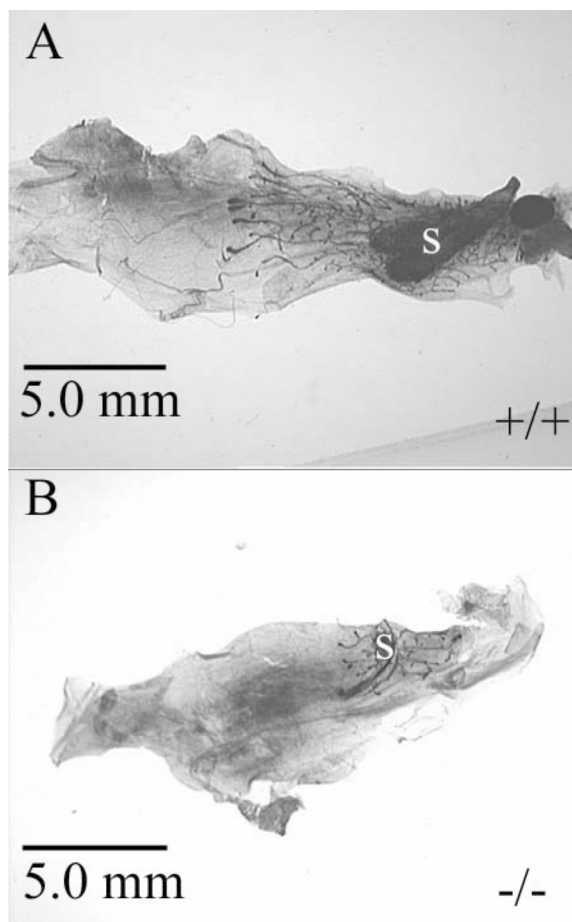


Figure 3. Comparison of mammary ductal development in hematoxylin-stained whole mounts of mammary tissue grafts established from the mammary rudiments of e16.5 *Igflr*^{+/+} (A) or *Igflr*^{-/-} (B) fetal mice. Grafts were placed into the cleared mammary fat pads of 3-wk-old female mice and allowed to develop for a period of 4 wk. After 4 wk, grafts were whole-mounted and stained with hematoxylin. Variable amounts of grafted skin (S) are also associated with the graft site. *Igflr*^{+/+} and *Igflr*^{-/-} = insulin-like growth factor I receptor genotype.

rdU cells within *Igflr*^{-/-} TEB but had no effect on apoptosis. In addition, the development of *Igflr*^{-/-} grafts was dramatically increased during early pregnancy compared with *Igflr*^{-/-} grafts allowed to develop for a similar length of time in nonpregnant hosts.

Results from the analysis of *Igflr*^{-/-} grafts supports the idea that stimulation of cell survival pathways by IGF-IR may not be as important to TEB development as stimulation of cell cycle pathways and that pregnancy-dependent proliferative pathways exist that can compensate for loss of the IGF-IR. In this respect, analysis of the phosphorylation state of proteins involved in cell survival such as Akt, or proteins involved in proliferation such as map-kinase or β -catenin, would be valuable. Current studies with the *Igflr*^{-/-} graft model are

applying immunofluorescence and confocal microscopy to analyze signaling protein activation in situ. Beyond the in situ approach, however, genetic crosses between *Igflr*^{-/-} mice and transgenic mice that display mammary-specific expression of cell cycle or apoptosis regulators should provide an unprecedented ability to map IGF-IR signaling within the mammary glands of intact animals. Noteworthy examples of success with this type of approach (Guy et al., 1996; Hutchinson et al., 2001; Yu et al., 2001) serve as indicators that much can be learned from the *Igflr*^{-/-} graft model about the regulation of mammary gland development by IGF-I.

IGF-I ACTION ON MILK SYNTHESIS

Cell Culture Models

The role of IGF-I in the stimulation of milk synthesis has been studied for several years in various model systems. Despite circumstantial evidence suggesting that IGF-I can mediate the effects of somatotropin on lactation, an unequivocal demonstration of this mechanism in all species, and under all experimental paradigms, is lacking. In cell culture models, clear evidence for the stimulation of milk protein gene expression by IGF-I has only been demonstrated in the mouse (Prosser et al., 1987) and rabbit (Duclos et al., 1989). In bovine mammary cell culture models, IGF-I clearly stimulates proliferation (Baumrucker and Stemberger, 1989), and can do so in an autocrine or paracrine fashion (Romagnolo et al., 1992), but is an ineffective galactopoietic agent (Peri et al., 1992).

Animal Models

Studies with whole animal models have also been equivocal with respect to the ability of IGF-I to stimulate milk synthesis. For example, in normal lactating goats, local infusion (Prosser et al., 1994) of IGF-I or IGF-II acutely increases milk synthesis in association with mammary blood flow (Prosser et al., 1990), but in transgenic mice or rabbits that overexpress IGF-I chronically in the mammary gland, little enhancement of milk yield was observed (Burrin et al., 1999; Wolf et al., 1997). The analysis of lactational capacity in mice that overexpress des(1-3)hIGF-I (**WAP-des**) within the lactating mammary gland has been conducted in two ways. First, the ability of WAP-des dams to support the growth of suckling pups was used as an indirect indicator of lactational capacity (Figure 4). This analysis demonstrated no growth advantage in litters suckled on WAP-des dams. In an independent experiment, the effects of the WAP-des transgene on lactational capacity were also found to be minimal, based on the evaluation of milk intake in 8-d-old pups suckled for 2

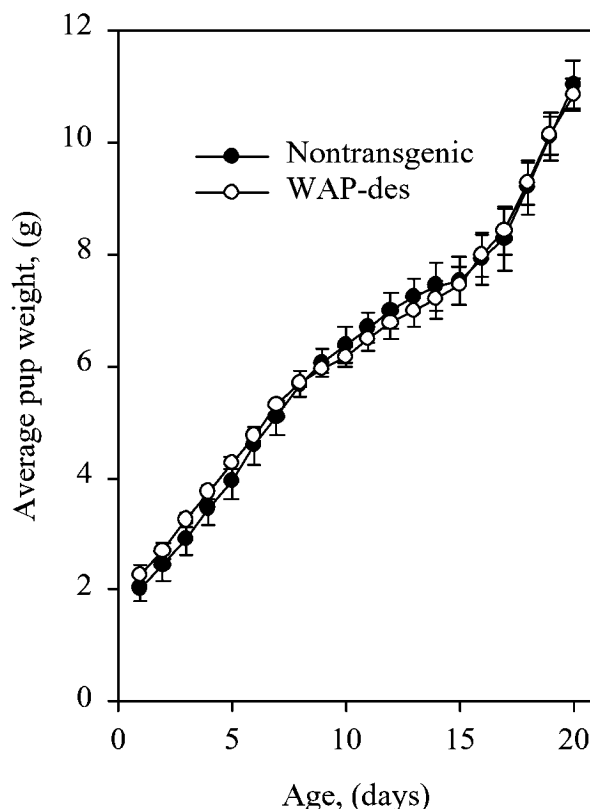


Figure 4. Overexpression of insulin-like growth factor I (IGF-I) within the mammary gland has no effects on lactation capacity. The BW of mouse pups suckled on either nontransgenic or WAP-des dams was monitored daily throughout the course of a normal lactation. Each symbol represents the mean \pm SEM for four and five nontransgenic and WAP-des dams, respectively. Average litter size were similar ($P > 0.05$) among nontransgenic and WAP-des dams at 7.5 ± 0.3 and 8.0 ± 0.3 , respectively. WAP = whey acidic protein, des = des(1-3)hIGF-I, hIGF = human IGF.

h after a 2-h fast (Burrin et al., 1999). In addition, concentrations of fat, protein, and lactose in milk from the WAP-des transgenic mice were similar to those found in nontransgenic mice (Table 1). These observations support the idea that exogenous IGF-I is probably incapable of directly stimulating milk synthesis in vivo.

Table 1. Protein, fat, and lactose concentrations in milk from 8-d lactating nontransgenic and whey acid protein-des (1-3)hIGFI.

	Nontransgenic	WAP-DES
Protein ¹ , mg/ml	171.0 \pm 16.4 ²	171.2 \pm 14.1
Fat, ³ %	17.3 \pm 0.9	17.2 \pm 1.9
Lactose, ⁴ mM	29.6 \pm 1.8	35.8 \pm 3.2
Sample number	6	4

¹Determined by microkjedahl.

²Means \pm SEM.

³Determined by Folch extraction.

⁴Determined by β -galactose dehydrogenase assay.

In fact, more recent observations with the WAP-des transgenic mice suggest that IGF-I may act by sustaining mammary cell number during late lactation (Hadsell et al., 2001) rather than stimulating milk synthesis.

IGF SERVE AS MAMMARY CELL SURVIVAL SIGNALS

Initial Observations

Anti-apoptotic actions of IGF-I have been described in several different cell types, and applications for these actions have been developed in the treatment of certain types of tumors and in the prevention of ischemia/reperfusion-induced apoptosis (Andrews et al., 2001; Yamashita et al., 2001). The idea that IGF-I regulates mammary gland involution was initially developed from the finding that IGF-I was a potent and somewhat specific inhibitor of apoptosis in several cell types in culture, including mammary cells (Geier et al., 1992; Harrington et al., 1994). Subsequent studies on involution of the prostate and mammary glands demonstrated dramatic increases in a negative regulator of IGF-I action, IGFBP5 (Tonner et al., 1995). The ability of IGF-I to actually delay mammary involution was demonstrated through targeted mammary-specific overexpression of IGF-I in transgenic mice (Hadsell et al., 1996; Neuenschwander et al., 1996). This ability was initially illustrated in two separate transgenic models. First, mammary whole mounts collected at 7 d after a normal 21-d lactation period from WAP-des mice showed much more alveolar development than that of nontransgenic mice (Hadsell et al., 1996). Second, TUNEL (terminal-deoxynucleotidyl-dUTP nick end labeling) staining of mammary gland specimens collected after 5 d of forced involution demonstrated reduced apoptosis in response to overexpression of either IGF-I or IGFBP3 (Neuenschwander et al., 1996). More recently, overexpression of IGF-II in the mammary gland has also been shown to inhibit apoptosis and delay mammary gland involution (Moorehead et al., 2001). Thus, the ability of overexpressed IGF-I or II to inhibit mammary involution in transgenic mouse models is clear, and overexpression of both of these growth factors causes mammary tumors (Bates et al., 1996; Hadsell et al., 2000). A potential limitation in these transgenic models is that the transgene-targeted IGFs are expressed only within the epithelium. Endogenous IGFs, predominantly expressed in the mammary stroma, are believed to stimulate the mammary epithelial compartment through a paracrine mechanism (Berry et al., 2001; Hovey et al., 1998; Walden et al., 1998). Thus, overexpression of IGF-I within the epithelium might be predicted to circumvent the normal paracrine signal and cause constitutive

activation of IGF-I signaling pathways in the epithelium. However, there may also be unpredicted consequences caused by altering the compartment in which the IGF-I is overexpressed. In addition, a distinction exists between the WAP-des mice and the other two models in that the overexpression of des(1-3)hIGF-I in the WAP-des models was observed to inhibit apoptosis only during natural mammary involution.

Forced or Natural Involution

In mice, milk yield peaks at 10 d postpartum, and then dramatically declines from d 16 to 20 postpartum, unless milk removal and suckling stimulus are maintained by cross-fostering young pups onto the dam in place of the older weanlings (Shipman et al., 1987). This decline in milk yield is associated with increased apoptosis and the eventual remodeling of the mammary epithelium in a process that has been called natural involution (Quarrie et al., 1996). Natural involution differs from the commonly studied forced involution in that it occurs at about half the rate of the latter (Hadsell et al., 2001) and is associated with sustained elevation in milk protein gene expression (D. L. Hadsell, unpublished observations, 1997). The initial observations with regard to the WAP-des transgenic mice were made at a time in which the mammary gland underwent natural involution (Hadsell et al., 1996). Subsequent experiments (Figure 5) demonstrated that apoptosis in WAP-des transgenic mice during natural involution could be reduced to as little as 50% of that observed in nontransgenic mice (Hadsell et al., 2000). These studies also demonstrated that natural involution could be delayed by cross-fostering young pups onto the dam before d 18 postpartum and that in these cross-fostered dams, overexpression of des(1-3)hIGF-I also reduced apoptosis (Figure 5). Despite these results, no inhibition of apoptosis could be detected in WAP-des mammary tissue during forced involution. This was surprising considering published results that show that both IGF-I and II could inhibit apoptosis during forced involution (Moorehead et al., 2001; Neuenschwander et al., 1996). An obvious explanation for the difference between the WAP-des mice and the other two models is the ability of the transgene products to interact with IGFBP (Clemmons et al., 1992). There were also apparent differences between the WAP-des mice and the IGF-II transgenics in the extent of **IRS-1** and Akt phosphorylation, with higher levels reported in response to overexpression of IGF-II. This could be related to the fact that IGF-II interacts with IR-A as well as the IGF-IR. However, establishing a relationship between differences in signaling pathway activation by the different IGF and protection from apoptosis during forced involu-

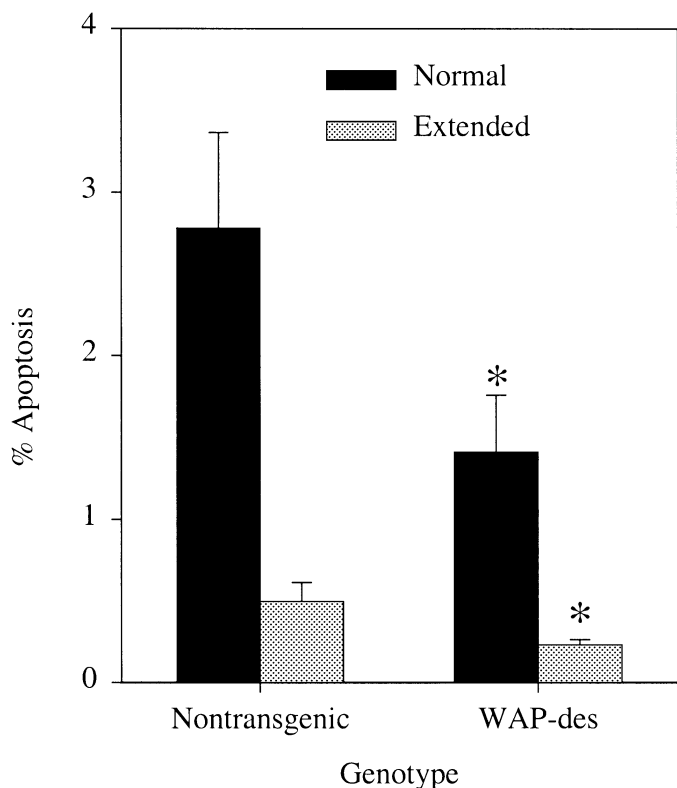


Figure 5. Overexpression of insulin-like growth factor I (IGF-I) inhibits mammary cell apoptosis during late lactation. Mammary tissue was collected at d 20 of lactation from nontransgenic or WAP-des dams during either normal or extended lactation. Lactation was extended by cross-fostering 10-d-old pups onto the dams at d 17 of lactation. Each bar represents the mean \pm SEM for three to seven dams. *Significant reduction compared with nontransgenic dams under the same conditions ($P < 0.05$). WAP = whey acidic protein, des = des(1-3)hIGF-I, hIGF = human IGF.

tion would require further comparison of the three models.

Signaling Cell Survival

A more detailed analysis of forced and natural involution in the WAP-des mice provided two pieces of evidence supporting that down-regulation of IGF-I signaling proteins may have precluded inhibition of apoptosis during forced involution (Hadsell et al., 2001). First, measurement of des(1-3)hIGF-I protein in mammary tissue of WAP-des mice demonstrated that the abundance of the transgene protein was similarly high during both natural and forced involution. Second, measurement of IGF-I signaling proteins in mammary tissue extracts demonstrated that induction of forced involution caused a relatively rapid and dramatic loss of the IGF-I-dependent signaling proteins, IRS-1 and 2 (Figure 6). This loss was associated with a decrease in the extent of phosphorylation of the serine/threonine

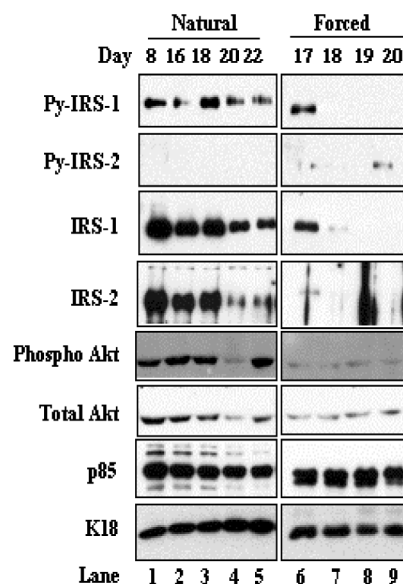


Figure 6. Analysis of insulin receptor substrate (IRS) and Akt expression and activation during natural and forced involution. Lactating FVB mice were placed with litters of 10 pups each at 2 d postpartum. At 16 d, postpartum mice were randomized to either natural or forced involution. For natural involution, the dams were left with the pups and killed for tissue samples on d 16, 18, 20, and 22 postpartum. Forced involution was induced at d 16 by removal of the pups, and mammary tissue samples were collected at d 17, 18, 19, and 20 postpartum. Tyrosine phosphorylation of IRS-1 (py-IRS-1) and IRS-2 (py-IRS-2) was determined in pooled mammary tissue extracts. Each pool represents four individual mice. Immunoprecipitation was conducted with 500 μ g of protein by using 5 μ g of antibody to IRS-1 or IRS-2. Phosphotyrosine was detected in these immunoprecipitates by Western blotting with antiphosphotyrosine antibody. Western blotting was also conducted with 30 μ g of protein from the same pooled extracts for total IRS-1 and IRS-2, p85 subunit of PI3 kinase, phospho-Akt, total Akt, and keratin 18. Data from natural involution are shown in lanes 1 through 5. Data from forced involution are shown in lanes 6 through 9. Figure derived from data of Hadsell et al. (2001).

kinase Akt. Recently published observations demonstrate that transgenic overexpression of constitutively activated forms of Akt within the mammary gland inhibit apoptosis during forced involution (Hutchinson et al., 2001; Schwertfeger et al., 2001). These observations support the idea that the maintenance of mammary epithelial cells during lactation depends on the integration of various inputs into a central cell survival pathway involving IRS and Akt. The importance of IRS-1 to maintenance of mammary cells during lactation is currently supported only by correlation. However, analysis of lactation and mammary involution within mice that carry a targeted mutation of the IRS-1 gene, as well as in transgenic mice that overexpress IRS-1 within the mammary gland, should determine the importance of this signaling protein to the mammary cell survival pathway.

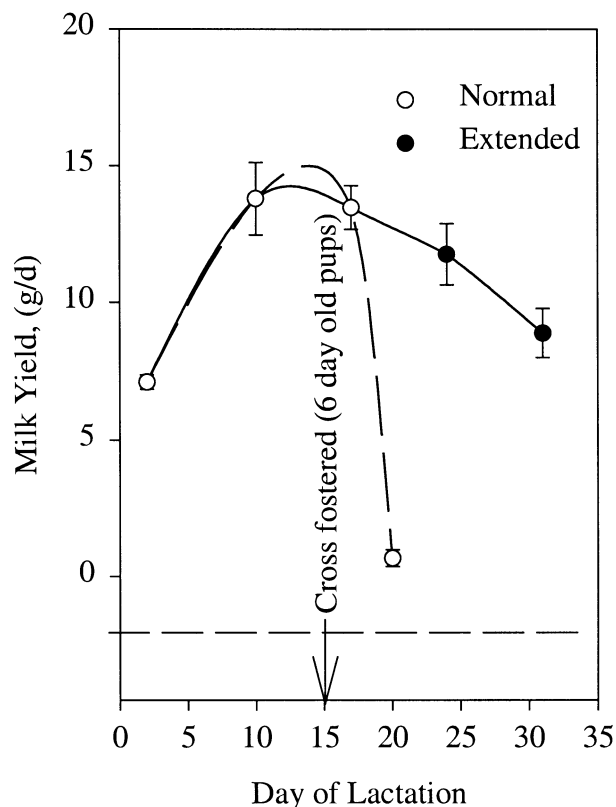


Figure 7. Extension of lactation in the mouse. Milk yield estimates by using $^3\text{H}_2\text{O}$ dilution in mice during either normal lactation or extended lactation. Data are from Shipman et al. (1987).

IGF-I and Lactational Persistence

Beyond providing motivation for the creation and analysis of new lines of transgenic mice, studies involving WAP-des mice have helped change the way in which IGF-I is thought to act within the lactating mammary gland. The ability of WAP-des to inhibit apoptosis during natural involution and slow the loss of mammary tissue supports a novel hypothesis that overexpressed IGF-I may also possess the capacity to enhance lactational persistence. Lactational persistence, though something of immense importance to the dairy industry, has been little studied in mouse models. Lactation in the mouse has been extended by several days through a single round of cross-fostering (Figure 7) and by up to 61 days through multiple rounds of cross-fostering (Nagasawa and Yanai, 1976; Shipman et al., 1987). These studies have demonstrated that despite continued suckling stimulus and milk removal, milk yield and mammary nucleic acids content declines over time. Preliminary studies conducted in our own laboratory find that repeated cross-fostering of 6-d-old pups onto a lactating dam at 6-d intervals allows for lactation to be extended to 50 d (D. L. Hadsell and D. Torres,

unpublished results, 2001). As with previous reports, lactational capacity and mammary gland DNA content declines during extended lactation. In addition, the ability of cross-fostering to inhibit mammary involution and extend lactation is clear from the observation that a single round of cross-fostering dramatically reduces mammary cell apoptosis (Figure 5). That this apoptosis is further reduced by overexpression of des(1-3)hIGF-I further supports the idea that overexpressed IGF-I can enhance lactational persistence (Figure 5). This hypothesis will be directly tested by using the extended lactation protocol discussed above. In addition, the extended lactation model should serve as a useful tool for identifying cellular and biochemical changes associated with the declining phase of lactation.

Milk yield is known to be a function of both the number of mammary secretory cells and synthetic activity per secretory cell (Capuco and Akers, 1999). In this respect, a normal lactation is characterized by cellular proliferation during the early stage, followed by dramatically diminished cellular proliferation and gradual cell loss as milk yield declines after peak lactation (Knight and Wilde, 1993) (Figure 8). The hypothesis of cellular senescence has been put forth as a means to explain the decline in milk yield during late lactation (Capuco and Akers, 1999). This hypothesis assumes that as they grow older, mammary epithelial cells lose their ability to synthesize milk and to proliferate, so that secretory cell number declines during later stages of lactation. Immunohistochemistry for casein has demonstrated that the decline in milk yield during lactation is associated with loss of synthetic ability of individual alveolar cells (Li et al., 1999). In addition, the cell loss that occurs during the declining phase of lactation is now known to be caused by apoptosis (Li et al., 1999). These observations imply that mammary cells wear out over time. Hence, the decline in lactation could be viewed as a degenerative process.

In other organ systems, increased apoptosis is associated with aging and a variety of related degenerative conditions (Higami and Shimokawa, 2000), and strong links exist among mitochondrial function, the accumulation of oxidative damage, and cellular apoptosis (Sastre et al., 2000). In addition, both IGF-I and Akt are capable of inhibiting apoptosis in response to acute oxidative damage *in vivo* (Daemen et al., 1999; Fujio et al., 2000; Yamashita et al., 2001). Cellular aging and oxidative stress within the context of the lactating mammary gland comprise a little-studied phenomenon. However, increased oxidation of glutathione in association with oxidative damage to mitochondrial DNA has been found to occur in the mammary gland during forced involution (Esteve et al., 1999). Based on this observation, it is reasonable to suspect that oxidative

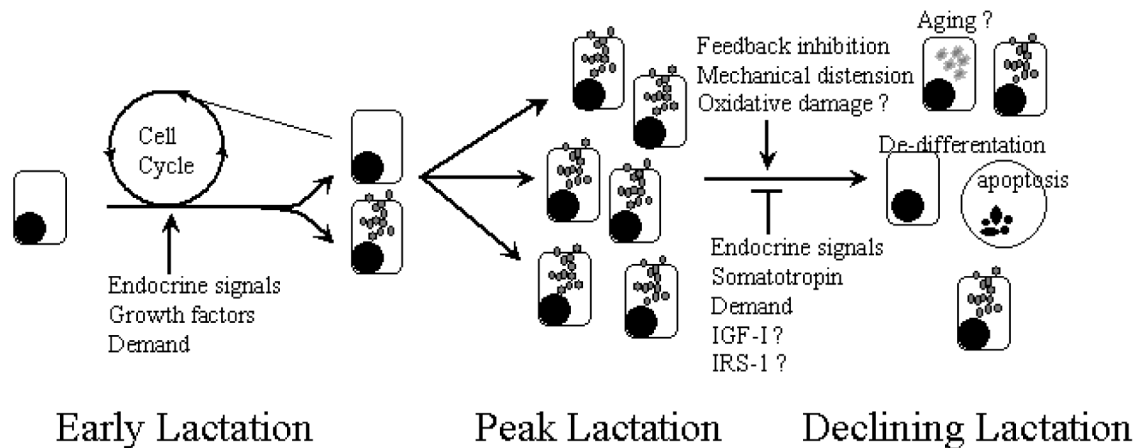


Figure 8. A model describing cellular changes over the course of a single lactation. Proliferative signals increase the number of epithelial cells during early lactation in response to milk demand, endocrine signals, and growth factors. This proliferative burst is thought to give rise to the population of milk secreting cells that are present at peak lactation. Decreased milk secretion during declining lactation is associated with increased apoptosis and decreased expression of milk proteins. Stimulators (→) and inhibitors (T-bar) of each transition are proposed.

damage may also play a role in cellular apoptosis during the declining phase of lactation, although on a slower time scale. Thus, overexpressed IGF-I might be hypothesized to enhance lactational persistence through an ability to ameliorate the effects of oxidative stress.

CONCLUSION

The ability to manipulate the mouse genome has resulted in the production of incredibly powerful tools for the study of mammary gland biology. The power of these tools lies in the ability to directly manipulate the expression and actions of specific gene products in a tissue-specific fashion. In gene knockout mice, the use of mammary gland transplantation has allowed the unprecedented evaluation of gene products whose biology would otherwise present a difficult challenge. Combining transgenic models with knockout models in the form of genetic crosses has enabled the functional dissection of cell signaling pathways *in vivo*. Data obtained from the analysis of several mouse models suggest that both the nature and the mechanism of IGF-I action on the mammary gland are very much dependent on physiological state. This was illustrated by the finding that loss of the IGF-IR during virgin mammary gland development affected cell proliferation and not apoptosis, whereas overexpression of IGF-I during lactation appeared to affect apoptosis rather than cell proliferation. The mechanisms underlying differential actions of IGF-I on the mammary gland remain to be determined. However, this goal will be attainable with the study of novel mouse models in which specific IGF-I signaling molecules are perturbed. In addition, the application of

transgenic models to understanding the regulation of milk yield has forced a reevaluation of current thought on IGF-I action during lactation. This has led to the proposal of a novel means for more directly relating observations collected in mouse models to an area of importance to the dairy industry, *i.e.*, regulation of lactational persistence. The application of the extended lactation paradigm to the analysis of both existing and novel strains of transgenic and knockout mice shows great promise for furthering our understanding of the regulation of milk yield during the declining phase of lactation.

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