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ABSTRACT

THE REGULATION OF STAT5A EXPRESSION AND ITS FUNCTION IN THE DEVELOPING MOUSE MAMMARY GLAND

By

Sarah Jane Santos

Signal transducer and activator of transcription (Stat)5a is a critical regulator of proliferation, differentiation and survival in mammary epithelial cells, and has been implicated in human breast cancer. While the pathways leading to Stat5a activation have been clearly elucidated, the mechanisms which control its expression are not well understood. To investigate potential regulators of Stat5a expression, an immunohistochemical analysis of Stat5a levels throughout mammary gland development was performed. The pattern of Stat5a expression suggested that it was regulated by the ovarian hormones, estrogen (E) and progesterone (P). This was confirmed through hormone ablation and reconstitution studies, where ovariectomy led to a severe decrease in Stat5a levels and treatment with E+P was necessary to restore its expression. Further studies demonstrated that Stat5a is expressed predominantly in estrogen receptor and progesterone receptor positive cells, suggesting that E and P directly induce the expression of Stat5a through their respective receptors.

Although Stat5a was found to be present and activated in the pubertal mammary gland, its functions outside of pregnancy and lactation are unknown. To investigate the role of Stat5a in early mammary gland development, a Stat5a knockout model was used. Aduclt nulliparous Stat5a-/- mice displayed defects in primary branching and side-

branching, as well as a diminished proliferative response to E+P treatment.

Having identified a role for Stat5a in E+P stimulated proliferation, the underlying mechanism by which it was acting was studied. Double labeling experiments indicated that Stat5a positive cells rarely proliferate, suggesting its actions are via a paracrine mechanism. Receptor activator of NF-κB ligand (RANKL), a secreted factor that can induce mammary epithelial cell proliferation, was demonstrated to be a downstream mediator of Stat5a. In addition, the nuclear localization and thus function of Id2, a critical RANKL target, was defective in Stat5a-/- mammary cells.

In summary, these studies reveal three novel findings concerning Stat5a in the mouse mammary gland. First, they demonstrate that Stat5a expression is controlled by E+P in the mammary gland. Secondly, a role for Stat5a in primary branching and side-branching in the virgin gland is shown. Finally, these studies establish a potential paracrine mechanism by which Stat5a induces proliferation via RANKL.

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TABLE OF CONTENTS

LIST OF FIGURES viii

CHAPTER 1

Literature Review

Anatomy of the mammary gland....2
Mammary gland development....4
Systemic mammogens: steroid hormones....9
Systemic mammogens: peptide hormones....13
Locally acting mammogens....15
Signal transducer and activator of transcription (Stat)....19
Stat structure....20
Stat pathway and regulation....21
Stat function: normal and disregulated....26
Stat5 expression in the mammary gland....27
Stat5 function in the mammary gland....29
Stat5 regulated genes....33
Stat5 and breast cancer....34
Conclusion....35
References....37

CHAPTER 2

Estrogen and Progesterone are Critical Regulators of Stat5a Expression in the Mouse Mammary Gland

Abstract....53
Introduction....54
Materials and Methods....56
Results....60
Discussion....79
References....85

CHAPTER 3

Examining the Function of Stat5a in the Virgin Mammary Gland and its Role in E+P Induced Proliferation

Abstract....90
Introduction....91
Materials and Methods....93

Results.....97
Discussion.....110
References.....117

Concluding Remarks.....120

APPENDIX

Regulation of Stat5a Expression by R5020 in Primary Mammary Epithelial Cells.....122

LIST OF FIGURES

CHAPTER 1

Figure 1.1: Cross section of a mammary gland duct.....3

Figure 1.2: Schematic presentation of the different stages of mammary gland 6
development

Figure 1.3: Basic overview of the Jak/Stat signaling pathway.....22

CHAPTER 2

Figure 2.1: Immunofluorescent staining of Stat5a during mouse mammary gland.... ...62
development

Figure 2.2: Stat5a expression in hormone treated mice.....65

Figure 2.3: Cellular localization of Stat5a in bromocriptine treated mice.....68

Figure 2.4: Colocalization of Stat5a with PRA or PRB.....71

Figure 2.5: Colocalization of Stat5a with ERα.....73

Figure 2.6: Immunodetection of Stat5a and BrdU in mammary glands of hormone.....76
treated mice

Figure 2.7: Immunodetection of Stat5a and WAP or Stat5a and RANKL.....78

CHAPTER 3

Figure 3.1: Whole mount analysis of pubertal and adult virgin mice.....99

Figure 3.2: Morphological response of mammary glands to hormone treatment.....101

Figure 3.3: Proliferative response of mammary glands to hormone treatment.....104

Figure 3.4: RANKL expression in response to hormone treatment.....107

Figure 3.5: Progesterone receptor expression in response to hormone treatment.....109

Figure 3.6: Id2 localization in response to hormone treatment 111

Figure 3.7: Proposed mechanism of Stat5a mediated proliferation in response 115
to E+P

APPENDIX

Figure 4.1: Stat5a mRNA expression in response to R5020.....127

CHAPTER 1

LITERATURE REVIEW

LITERATURE REVIEW

Anatomy of the mammary gland

The mammary gland presents a unique and fascinating system to study because the majority of its development and differentiation occurs postnatally. Unraveling the basic principles of mammary development is not only interesting, but is also central to our understanding of breast cancer. According to the American Cancer Society, 40,910 breast cancer related deaths will occur this year. A comprehensive knowledge of normal mammogenesis will aid our understanding of how mammary cells become deregulated in cancer, and may ultimately lead to developing new therapeutics.

The mammary gland is an evolutionarily young organ that secretes milk to sustain newborn offspring. In most mammals, the milk is delivered through a complex ductal system to the nipple. In contrast, monotremes have mammary glands without a central ductal system or nipple, and instead secrete milk into a milk patch (1). The discussion in this dissertation will focus on the former glandular system. Historically, the mouse has been favored as a model of both mammary gland development and breast cancer biology. In contrast to humans which have a single pair of glands, mice have five pairs of mammary glands which form along the entire trunk (2). Murine glands contain much less fibrous tissue (3), and do not produce structures to the degree of complexity seen in the human gland (2). Despite these differences, the mouse can still serve as an important model for the investigation of basic mammary gland biology.

The mammary gland is composed of two distinct compartments, each containing multiple cell types (Fig. 1.1). The epithelium makes up the secretory component of the

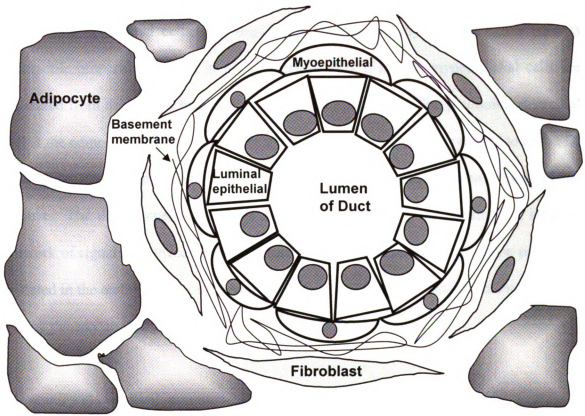

Figure 1.1. Cross section of a mammary gland duct. Secretory luminal epithelial cells surround the lumen of the duct, and are apically located to a single layer of contractile myoepithelial cells. Myoepithelial cells are surrounded by a basement membrane. In the stroma, fibroblasts surround the basement membrane, and adipocytes fill the remainder of the fat pad. Adaptation of figure is used with permission from Alexis Drolet.

organ, while the supporting stroma surrounds the epithelium (4). The two major epithelial cell types are luminal and myoepithelial. The cuboidal luminal cells line the ducts and alveoli, and are polarized and secretory in nature. Myoepithelial cells, also referred to as basal cells, are elongated in shape and form a continuous layer surrounding the outside of luminal cells. As implied by their name, myoepithelial cells possess contractile properties that aid in the delivery of milk (5). The myoepithelial cells are separated from the stroma by a layer of basement membrane. The predominant stromal cell is the adipocyte, and for this reason the mammary stroma is also referred to as the fat pad. Fibroblasts, endothelial cells, immune cells and neurons are also present in the stroma. The development of a fully functional mammary gland requires an intricate network of signaling between the epithelium and stroma. This communication process is initiated in the embryo and continues through two major stages of development that occur during puberty and pregnancy.

Mammary gland development

The mammary gland is derived from the epithelial cell lineage, and is produced as a result of an invagination of the dermis. The first evidence of development in most mammals is the milk line, an elevated ridge of ectoderm. The milk line then separates into raised, lens-shaped aggregates of epithelial cells at specific locations where mammary glands will form. In primates, these ectodermal placodes are produced in the thoracic region (6), whereas rodents develop them along the entire trunk. In the mouse embryo, this process can be observed as early as late embryonic day 10 (E10) to early E11 (7). The formation of the mammary placodes is not through the proliferation of

cells, but rather through the migration of epidermal cells (7, 8). Within one day, the placodes develop into mammary buds and are visible as elevated knob-like structures (9). By the following day, the mammary buds begin to invaginate into the dermis, and by E14.5, can no longer be detected externally (9). At approximately E15, cell proliferation occurs at the tip of the invaginated bud to form a duct, called the primary sprout. Each sprout forms a lumen, which opens on the surface of the skin, where the nipple forms. By E18.5, the sprout has elongated and bifurcated to produce approximately 6-8 short ducts, which comprise the rudimentary gland observed at partuition (9). This stage of development is hormone independent, and occurs primarily in response to signaling between the epithelium and mesenchyme. Several regulators of embryonic mammary gland (anlage) development have been identified, and it is interesting to note that many of these also have roles in breast cancer. Fibroblast growth factor (FGF)10, neuregulin (Nrg)3, parathyroid hormone related peptide (PTHrP) and Wnts have all been shown to be critical signalers in the formation of the mammary anlage (10).

The mammary anlage contains a population of stem cells with the capacity to give rise to all cell types of the mature mammary gland. In fact, mammary buds can be isolated and transplanted into cleared fat pads of recipient mice, to form fully developed mammary glands (11). This is an important technique that is used to examine the effects of deleting specific genes in mice when germ line deletion causes lethality.

After birth, the rudimentary gland grows isometrically with the rest of the body. The first stage of dynamic mammary development does not occur until puberty, when epithelial cell proliferation is initiated by the surge in ovarian hormones (see Figure 1.2).

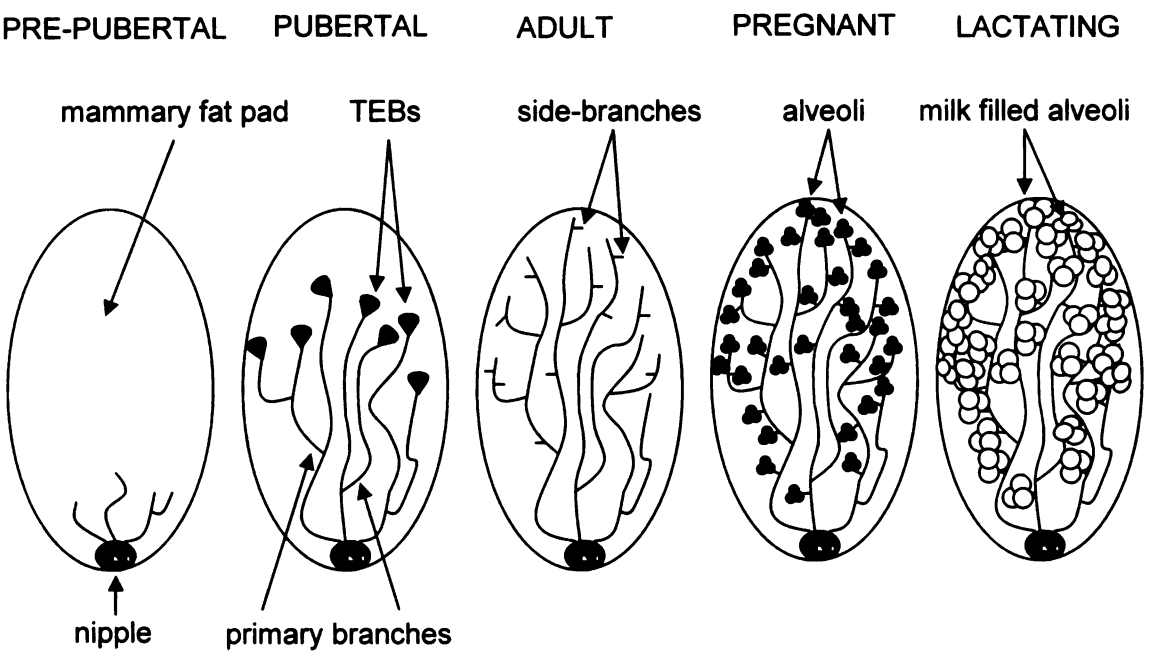

Figure 1.2. Schematic presentation of the different stages of mammary gland development. A rudimentary ductal system is present within the mammary fat pad at birth, and grows at the same rate as the animal until the onset of puberty. During puberty, the production of E and P by the ovaries promotes ductal elongation, and conspicuous club-shaped structures (terminal end buds (TEBs)), where the majority of epithelial cell proliferation occurs, appear at the duct ends. In the adult virgin, the entire fat pad is filled with a regularly spaced system of primary ducts, with side-branches that form and regress to some degree in each estrous cycle. The increase in hormones during pregnancy (estrogen, progesterone, prolactin, and placental lactogens) increase cell proliferation and induce the formation of alveoli. These alveoli expand and differentiate into milk-secreting structures at the end of pregnancy. In the lactating gland, fully mature luminal epithelial cells synthesize and secrete milk components into the alveolar lumina.

The production of estrogen (E) and progesterone (P) at puberty drives ductal elongation into the fat pad. The majority of proliferation occurs in prominent club-shaped structures located at the distal ends of ducts called terminal end buds (TEB)s (4). Two distinct cell types define TEB anatomy, body cells and cap cells (12). Multiple layers of body cells, which differentiate primarily into luminal cells, are enclosed by a single outer layer of cap cells, which give rise to myoepithelial cells (12). The body cells closest to the cap cell layer are highly proliferative and support the outward growth of the duct. Meanwhile, the body cells furthest from the cap cell layer undergo apoptosis to support the formation of a hollow lumen in the developing duct (13). Bifurcation and trifurcation of the TEBs causes dichotomous primary branching of the epithelium to produce an arborized ductal tree. When the perimeter of the fat pad is reached, TEBs regress and the majority of proliferation ceases at this stage until pregnancy (13).

The remaining proliferation observed in the adult virgin gland is manifested in the development of side-branches. These small structures branch off laterally from established primary ducts. Side-branches grow and regress intermittently in response to the rising and falling hormone levels during the estrous cycle (14). However, the side-branches never fully regress, resulting in ever increasing mammary gland complexity as the animal matures (14). In the mature mammary gland, primary ducts may be composed of several layers of luminal epithelial cells, which become fewer as the distance from the nipple increases. Most terminal ducts and side-branches have only one layer of luminal epithelial cells (15).

The second major stage of postnatal mammary gland development occurs during pregnancy and results in a fully functional gland. An increase in ovarian hormone levels,

particularly P, and the addition of placental hormones results in further ductal side-branching, and alveolar units subsequently form at the ends of these branches. Alveoli serve as the major milk-producing components of the gland. They are comprised of fully differentiated luminal epithelial cells surrounding a central cavity. Alveolar cells are polarized so that milk constituents are secreted from their apical membrane into the lumen. There are two phases of lactogenesis. The first occurs in mid to late pregnancy and is characterized by the expression of milk proteins. Lipid droplets are visible in the cytoplasm of luminal cells during this phase. In the second phase, which occurs near parturition, milk protein expression increases even further, and lipid droplets and proteins are secreted into the alveolar lumen. In addition to lipids and lactose, the major protein constituents of milk are casein, lactalbumin and whey acidic protein (WAP). In response to suckling, oxytocin is released and stimulates the contraction of myoepithelial cells surrounding alveoli and ducts, resulting in milk release. Cessation of lactation due to weaning induces a massive remodeling of the mammary gland called involution. During this period, alveolar cells undergo apoptosis and regress (16), resulting in a gland that closely resembles that of mature, virgin animals. The ability of the mammary gland to undergo the process of creating milk-producing alveoli with each successive pregnancy underlines the existence of mammary stem or progenitor cells. Further analysis is necessary to fully understand the roles and regulation of mammary stem cells in normal and malignant tissue development.

Systemic mammogens: steroid hormones

Several signaling networks are required for the development of a functional mammary gland, and systemic endocrine factors play a paramount role in this process. E and P are the principle steroid hormone regulators of post-pubertal mammogenesis. The control of mammary development by these ovarian hormones was initially revealed through endocrine ablation studies over 100 years ago, when Halban demonstrated that ovariectomy induced mammary regression (17).

E acts via binding to its two receptors, ERα and ERβ. Like other members of the steroid receptor super-family, when bound by their ligand, these receptors act as transcription factors in the nucleus, initiating the expression of specific responsive genes (18). In addition to this classical ER genomic action, a smaller amount of ER resides in the cell membrane or cytoplasm, where it can initiate more rapid responses through direct interactions with a variety of signaling pathways (19). ERα, but not ERβ, plays a primary role in the outgrowth of mammary ducts during puberty (20). ERα-/- mice do not produce TEBs, and the gland fails to develop past the prepubertal stage. Early transplant experiments using ERα-/- tissue to investigate if stromal or epithelial ERα was required for mammary gland development resulted in contradictory results. One study concluded that only stromal ERα was necessary for ductal outgrowth. This was shown by transplanting recombined mammary stromal and epithelial tissue from neonatal wild-type and ERα-/- glands under the renal capsule of nude mice (21). A separate study demonstrated that both epithelial and stromal ERα were required for complete mammary gland development in adult mice when mammary epithelial cells were isolated from adult ERα-/- or wild-type mice and injected into epithelial-free mammary fat pads (22). These

data suggest that mammary development in neonatal and adult mammary epithelial tissue is carried out via different ER mechanisms. However, all of these experiments were complicated by the fact that they used hypomorphic knockout mammary glands (23). Although full length ERα was not functional in these mice, alternative splicing gave rise to a small amount of truncated ERα product which retained some transactivation potential (24, 25). It has been recently shown using a new ERα-/- model, which has no detectable ERα transcript, that epithelial ERα, but not stromal ERα, is essential for ductal outgrowth in puberty (26). Much less is understood about ERβ function in the mammary gland. Unlike ERα, ERβ is not necessary for normal pubertal development, but instead appears to play a role in differentiation during pregnancy (27).

Like E, P's action is also mediated through its intracellular receptor, PR. There are two major PR isoforms, PRA and PRB, which are produced by a single gene through differential transcription from two initiation sites, and translation initiation at two alternative AUG initiation codons (28). These receptors exhibit different temporal and spatial patterns of expression in the mouse mammary gland (29). PRA is the predominant isoform present in the virgin gland, where it is expressed in a subset of epithelial cells located in a punctate pattern throughout the ducts. In the pregnant gland, PRA continues to be expressed in both ductal and alveolar cells, but the overall percentage of PRA positive cells and the amount of PRA protein per cell decreases as the pregnancy proceeds. In contrast, PRB is not detectable in the virgin gland, and is primarily confined to a subset of alveolar cells during pregnancy. Both isoforms become undetectable during lactation. Expression of both receptors reappears in the post-involuted gland, where PRA levels are similar to those seen in the late pregnant gland,

but PRB levels are lower. The two PR isoforms are rarely expressed in the same cell in the mouse mammary gland (29). It is interesting to note that the expression pattern of PR in humans and rats differs from the mouse in two ways. First, PRB is present in the nulliparous human and rat mammary gland, and secondly, PRA and PRB are often coexpressed in the same mammary epithelial cell in these species (30).

The role of P in mammary development was not examined in detail until recently, when studies using PR gene-deleted mice (total PR-/-, PRA-/- only or PRB-/- only) (31-33) and transgenic mice (PRA or PRB transgenes) (34, 35) were carried out. Whole mount analysis revealed that the ductal structure of the adult PR-/- mammary gland was similar to that of agematched wild-type mice. However, PR-/- mammary glands failed to respond to exogenous E+P treatment, displaying a lack of side-branching and alveolar development. Transplantation experiments have determined that the role of PR in alveologenesis during pregnancy or in response to E+P is intrinsic to the epithelium (36).

Ovariectomized PRA-/- mice showed normal side-branching and alveologenesis in response to E+P treatment, indicating that PRB is sufficient for this process (32). PRB-/- mice failed to develop side-branches or alveoli during pregnancy or when treated with E+P, (33) further demonstrating that it is the critical isoform for these developmental processes. Adult glands from PRA transgenic mice displayed an increased amount of side-branching, sometimes resulting in glands resembling those in early pregnant animals (34). In contrast, robust alveolar development with only limited side-branching, and an inhibition of ductal elongation was seen in glands from PRB transgenic mice (35). Thus, PR is not critical for ductal outgrowth during puberty, but

PRB is required for alveologenesis during pregnancy, and PRA may play a role in side-branching.

The glucocoriticoid receptor (GR) also participates in mammary development. Deletion of the GR gene results in embryonic lethality, so normal mammary development cannot be examined. To circumvent this problem, GR-/- outgrowths from embryonic mammary buds were transplanted into cleared fat pads of wild-type hosts (37). The resulting mammary glands displayed abnormal development during puberty with distended ductal lumina and multiple layers of ductal epithelial cells instead of the standard single layer. Another study demonstrated that mice expressing a GR that lacks DNA binding ability had decreased side-branching in the adult virgin mammary gland (38). *In vitro* experiments have established that glucocorticoids are lactogenic factors (39). However, the conditional deletion of GR during pregnancy did not affect milk production, but instead showed a reduction in epithelial cell proliferation (40).

The 1,25-(OH)2D3 (vitamin D3) receptor (VDR) is another member of the nuclear hormone receptor superfamily (41, 42), which participates in mammary development (43). Loss of the VDR has been shown to result in defective regulation of ductal outgrowth in the mouse mammary gland. After treatment to normalize fertility, calcium levels and E levels, VDR -/- mice exhibited accelerated ductal elongation and an increased number of TEBs during puberty (44). In addition, treatment of these mice with ovarian hormones *in vivo* revealed an increased proliferative response. Vitamin D3 appears to antagonize the proliferative signal from E+P in the pubertal mammary gland.

Systemic mammogens: peptide hormones

In the early twentieth century, hypophysectomy experiments revealed that factors other than ovarian hormones were necessary for mammary gland development (45). In 1928, Stricker and Grueter showed that milk secretion could be induced in virgin rabbits by injecting pituitary extracts from lactating animals (46). The pituitary derived factor responsible for the maturation of the mammary gland was isolated four years later and named prolactin (PRL) for its milk inducing attributes (47). PRL is primarily produced and secreted by specialized cells in the anterior pituitary gland called lactotrophs. E, and to a lesser extent P, induce the hypothalamus to secrete galanin (48), which in turn induces the lactotrophs to secrete PRL (49). During lactation, the suckling stimulus drives secretion of PRL from the pituitary (50). The hormone is also synthesized to a lesser extent locally in the mammary gland (51).

Unlike the steroid hormones E and P, PRL is a peptide hormone which acts through a transmembrane receptor belonging to the cytokine receptor superfamily (52). The mammary PRL receptor (PRLR) signals via the Janus kinase (Jak)2 protein to activate the signal transducer and activator of transcription (Stat)5a, mitogen-activated protein (MAP) kinase, and other signaling pathways. Several studies have used PRLR gene knockout mice to determine the role of PRL in the mammary gland. Adult virgin PRLR-/- glands exhibited decreased side-branching. However, transplantation studies showed that this defect was not intrinsic to the epithelium, but rather was due to the inability of PRL to stimulate P secretion from the ovaries (53-55). Since PRLR-/- mice are infertile, PRLR+/- mice were used to examine mammogenesis during pregnancy. Up to mid-pregnancy, mammary development was indistinguishable from wild-type mice.

However, a greater amount of alveolar development was noticeable in the wild-type females after day 15 of pregnancy, and failed to produce milk after parturition (54). Similar results were obtained when PRLR-/- epithelium was engrafted to wild-type stroma (55). These results demonstrate that PRLR-/- and PRB-/- mammary glands have similar developmental phenotypes. Thus, PRL is an important mitogen and differentiation factor in the pregnant mammary gland.

In addition to PRL, other PRL-like hormones are produced by the placenta during pregnacy. In mice and rats, identified PRL-related hormones now number more than a dozen. The first two of these hormones were initially revealed by searches for placental proteins in rodents that bind with high affinity to the PRL receptor and mimic the action of PRL (56, 57). These two hormones have been designated as "placental lactogens" (placental lactogen I (PL-I) and (PL-II)) reflecting their origin and roles as inducers of milk production. At mid-gestation, pituitary derived PRL becomes less necessary for mammopoiesis, as the placental lactogens increase in amount and take over as the major stimulatory factors (58, 59). After partuition, when PL-I and PL-II are absent, PRL becomes necessary for the maintenance of alveoli and production of milk. When the suckling stimulus is removed at weaning, and pressure from milk build up increases, the mammary gland undergoes involution (60).

Growth hormone (GH) is another factor secreted by the anterior pituitary gland that regulates mammary development. Mammary glands of mice lacking the GH receptor (GHR) gene exhibited delayed ductal outgrowth and restricted side-branching at puberty (61). Transplantation of GHR-/- mammary epithelium into cleared fat pads of wild-type mice showed that stromal GHR was sufficient for normal ductal outgrowth

(61). It is well accepted that GH and E are the principle hormones driving pubertal mammary development (62). More recent data suggests that GH also plays a role in both alveolar development and lactation. In PRLR+/- mice, treatment with GH during pregnancy was able to restore alveolar development (63). Other studies have shown that GH synergizes with PRL for normal milk production in rats (64, 65), and GH is able to stimulate milk protein expression in primary mouse mammary cells (66).

Locally acting mammogens

Early models of systemic E action in the mammary gland were based on the correlation of its ability to induce epithelial cell proliferation and the presence of ER in the epithelium. This led to the hypothesis that E induces proliferation directly in the ER expressing epithelial cells. However, the discovery that the majority of proliferating cells in the developing mammary epithelium do not contain steroid hormone receptors (67-69) complicated this initial model. Instead, the steroid receptor-positive cells are often in close proximity to proliferating cells which incorporate ^{3}H-thymidine (67). It is now known that locally produced growth factors mediate the proliferative effects of the mammogenic steroid hormones. Some growth factors are direct transcriptional targets of hormone receptors, and the germline deletion of the genes encoding these growth factors or their receptors results in mammary phenotypes similar to those in knockout models of the steroid hormone receptors which control their expression.

The first example of a paracrine factor mediating the effect of a systemic mammogen was revealed by the finding that insulin-like growth factor 1 (IGF-1) could reproduce the effects of GH in promoting mammary gland development, and that GH

acted on the stromal compartment to increase IGF-1 mRNA levels (70-72). IGF-1-/- and GHR-/- mammary glands exhibit similar defects in pubertal ductal outgrowth, suggesting that they act in a common pathway (61, 73). Treatment with IGF-1 + E was able to rescue the defects seen in IGF-1-/- mice, while GH + E treatment could not (73). This confirmed that IGF-1 is downstream of GH. Although GH or IGF-1 alone could induce some mammary development in ovariectomized, hypophysectomized, pubertal animals, the addition of E was required to achieve full mammary development, suggesting a synergism of these two signaling pathways (74, 75). It was later demonstrated that mice with a liver-specific deletion of the IGF-1 gene had normal mammary IGF-1 transcript levels and normal mammary development at puberty despite having significantly reduced serum IGF-1 levels (76). This finding demonstrates that only locally produced IGF-1 is necessary for ductal outgrowth.

PRL induced proliferation is also accomplished via paracrine signaling in the mammary gland. One mediator of this PRL activity is IGF-2. The first evidence that IGF-2 was involved in PRL's effects came from studies where ectopic expression of IGF-2 was shown to restore alveologenesis in pregnant mammary glands derived from PRLR-/- epithelial cells transplanted into wild-type stroma (55). Consistent with this notion was the observation that transplanted IFG-2-/- epithelial cells exhibit a similar defect in alveolar development as PRLR-/- cells (55). Finally, PRL treatment was able to induce IGF-2 mRNA expression in primary mammary epithelial cells, and IGF-2 treatment of primary cells resulted in an increase in cyclin D1 protein (55). Taken together, these observations suggested that PRLR is required for the synthesis of IGF-2 by mammary epithelial cells and that once produced, IGF-2 can act in a paracrine manner to induce

cyclin D1 expression. This pathway is similar to that of the GHR, the closest relative of the PRLR, and its paracrine mediator IGF-1, except that PRL effects are intrinsic to the epithelium.

Epidermal growth factor receptor (EGFR) is a critical downstream mediator of E induced ductal elongation and branching in the pubertal mammary gland. The EGFR is a member of the ErbB/type1 family of receptor tyrosine kinases, and forms homodimers or heterodimers with the other family members, ErbB2, ErbB3 and ErbB4 (77). Multiple ligands bind to the EGFR, including but not exclusively EGF, transforming growth factor-α (TGF-α), and amphiregulin (AR) (78). The function of EGFR in mammogenesis was demonstrated using two mouse models, one with targeted expression of a dominant negative EGFR in the mammary gland (79) and a second in which neonatal EGFR-/- mammary glands were transplanted into wild-type hosts (80). Both of these models exhibited defective mammary ductal development at puberty. The transplant experiments demonstrated that stromal, but not epithelial EGFR was essential for pubertal ductal outgrowth (80). Further experiments have shown that ErbB2 also contributes to pubertal ductal outgrowth, but unlike EGFR, ErbB2 acts primarily in the epithelium (81).

E treatment stimulates tyrosine phosphorylation and activation of both EGFR and ErbB2 in ovariectomized animals at puberty (82). This is likely due to the fact that expression of AR, the chief EGFR ligand during puberty, is regulated directly by E (83). AR mRNA is expressed at higher levels than other EGFR ligands during puberty and is synthesized by ductal and TEB epithelial cells (84). AR-/- mice exhibit blunted ductal outgrowth at puberty, similar to ERα-/- mice, and implanted AR-secreting pellets

promote ductal outgrowth (84). Taken together, these data suggest a model in which E acting through epithelial ER, induces AR synthesis and secretion, and the secreted AR then acts on stromal EGFR and epithelial ErB2 to induce ductal outgrowth. The downstream responses to AR action in stromal cells remain to be explored. In the simplest scenario, stromal cells stimulated by AR could emit a proliferative signal that is received by the neighboring epithelial cells. Several growth factors, such as hepatocyte growth factor (HGF), IGF-1, and FGF10, are good candidates because they are expressed in the mammary stroma at the time of ductal elongation, whereas their respective receptors are found in the epithelium (85).

The proliferative action of P through PR in mammary epithelial cells is also paracrine in nature. This was first revealed by the observation that wild-type epithelium, within close proximity of PR-/- epithelium, could mediate alveolar formation in the knockout tissue (36). Wnts were proposed as likely mediators of P-induced side-branching after ectopic expression of Wnt1 was shown to overcome the defect in PR-/- glands (86). However, Wnt1 is not normally expressed in the mammary gland. Instead, Wnt4, which is expressed in the early to mid pregnant gland, was demonstrated to be critical for side-branching and was induced by P *in vivo* (86). To date, studies have only implicated Wnts in the side-branching which mostly occurs during pregnancy.

Another paracrine mediator of hormone induced mammary development is the TNF family member, receptor activator of NF-kB ligand (RANKL), also known as OPGL, ODF, and TRANCE (87). Although RANKL is primarily known for its functions in bone remodeling and immunity, it has been shown recently that RANKL is also a key regulator of proliferation during mammary gland development (87). Pubertal and

nulliparous adult mice lacking RANKL or its receptor, RANK, show normal mammary gland morphology (88). However, during pregnancy, RANKL-/- mice display impaired alveolar formation, and mammary tissue transplant experiments showed that the effect of RANKL was intrinsic to the epithelial compartment (88). Overexpression of RANK in the mammary gland resulted in an increase in proliferation and a decrease in differentiation during pregnancy (89). Thus, RANKL is a critical mitogen in the pregnant mammary gland.

RANKL expression is controlled by a number of factors. P, PRL and PTHrP, all of which are upregulated during pregnancy, have all been shown to induce the expression of RANKL in the mammary gland (88). This is consistent with the fact that RANKL expression is not detectable by Western blotting in the nulliparous adult gland, but is increasingly expressed as pregnancy progresses (88). In contrast, its receptor RANK is constitutively expressed in the mammary gland throughout development (88).

Signal transducer and activator of transcription (Stat)

The signal transducer and activator of transcription (Stat) signaling pathway is critical for normal mammary gland development. This pathway transmits extracellular signals received by transmembrane receptors to the nucleus, resulting in specific genetic responses. In their inactivated form, latent Stats are located in the cytoplasm. Following receptor activation, Stats translocate to the nucleus and directly induce transcription of responsive genes. Hence they act as signal transducers in the cytoplasm and transcription factors in the nucleus. This pathway allows for an efficient transcriptional response to external signals without the need for secondary messengers.

Stat structure

Stats are evolutionarily conserved, and are found in eurkaryotes ranging from slime molds to humans (90). In mammals, seven Stat proteins (Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6) are transcribed from separate genes. These proteins range in size from 750 and 850 amino acids (91). To date, no crystal structure of a whole Stat molecule has been solved. Most of what is known about the three-dimensional structure stems from crystallographic studies of specific domains from Stats 1, 3 and 4 (91).

Stat proteins are defined by the presence of five conserved domains. The amino-terminal half of the protein consists of two relatively under-characterized domains. The first domain consists of ~125 amino acids, and is reported to aid in DNA binding and to regulate nuclear translocation (92, 93). There is also a coiled-coil domain (amino acids ~135 to ~315) comprised of a four-helix bundle, which functions in protein-protein interactions (90). The domains that compose the carboxy-terminus are better characterized. The DNA-binding domain (DBD) (amino acids ~320 to ~480) binds to members of the gamma activating sites (GAS) family of enhancers, and also appears to regulate nuclear export (94, 95). The structure of the Stat1 or Stat3 DBD bound to DNA consists of several beta sheets, and is similar in configuration to domains of other transcription factors such as p53 and NF-κB (96). Adjacent to the DBD is a linker domain (amino acids ~480 to ~575), which is important in the stability of the Stat-DNA interaction (97). A Src homology 2 (SH2) domain (amino acids ~575 to ~680) is the most highly conserved motif and is critical for both the recruitment of Stats to receptors and Stat dimerization (92). Finally, the carboxy-terminus contains a transcriptional

activation domain (TAD), which varies considerably in both length and sequence, conferring specificity to the Stat family members (98).

Stat pathway and regulation

A large variety of cytokine and growth factor (polypeptide ligands) can induce Stat activation. When these ligands bind their cognate receptor, they induce the dimerization or oligomerization of the receptors. This leads to the apposition of the associated Jaks, which are non-covalently bound to the cytoplasmic tail of receptors (99) (see Figure 1.3). Once within close proximity, transphosphorylation of the Jaks occurs on tyrosine residues. The activated Jaks subsequently phosphorylate tyrosines within the cytoplasmic domains of the cytokine-receptor, providing docking sites for the SH2 domains in Stat proteins. The Stats are then recruited to the receptor complex, whereupon they are phosphorylated on a single tyrosine residue near their carboxy terminus by the Jaks (100) . Alternatively, Stats can be directly phosphorylated and activated by receptor tyrosine kinases such as the EGFR, colony stimulating factor-1 receptor (CSF-1R), and platelet derived growth factor receptor (PDGF-R) (92). The non receptor tyrosine kinase Src can also phosphorylate Stat proteins (101). Regardless of how they are phosphorylated, Stat dimerization then occurs through reciprocal phosphotyrosine–SH2 interactions, and released from the receptor complex (102). Phosphorylated Stat monomers have never been detected, and it is speculated that this is because two Stats are activated simultaneously within such close proximity that dimerization occurs instantaneously.

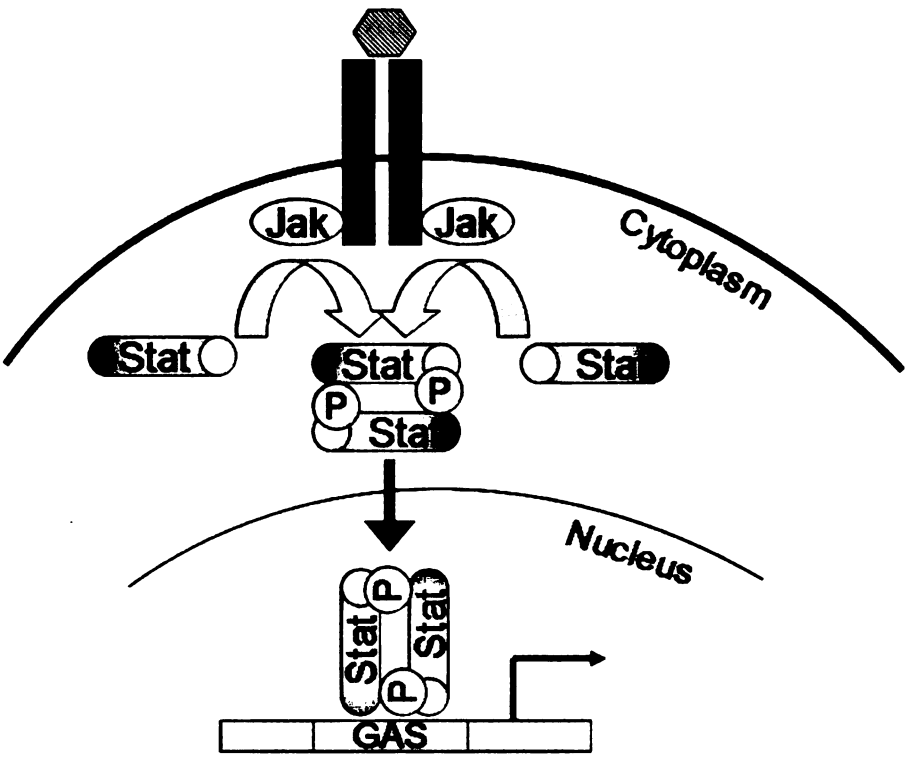

Figure 1.3. Basic overview of the Jak/Stat signaling pathway. Upon ligand binding, cytokine receptors dimerize, bringing their associated Jaks within close proximity to each other. Once activated by transphosphorylation, the Jaks phosphorylate tyrosines on the receptor cytoplasmic tail. Stat proteins are then recruited to the phospho-tyrosines via their SH2 domains. Jaks phosphorylate specific tyrosine residues within recruited Stat proteins, and phosphorylated Stats dimerize and release from the receptor complex. Stat dimers translocate to the nucleus and bind to members of the GAS family of enhancers to induce transcription of Stat regulated genes.

Once dimerized, nuclear localization signals are revealed, and Stats quickly translocate to the nucleus (91). Due to the large size (~180 kDa) of these complexes, Stat dimers require the assistance of an α-importin protein to enter the nucleus through the nuclear pore complex (103). When Stats are bound by the importin inside the nucleus, their DNA-binding site is masked (104, 105). However, competition with specific DNA sequences dislodges the importin, since these sequences have a higher binding affinity for Stat DBDs (104).

The major effects of activated Stats are through their ability to induce transcription of target genes. Phosphorylated Stat proteins bind to gene promoters containing GAS enhancer elements (TTCNNNGAA) (94). The specificity for gene expression induced by the different Stat proteins is attained through their varying binding affinities for target DNA sites, and variations in the nucleotide sequence (106). Stats bind to the DNA directly, and associate with other transcription factors and co-activators to induce gene expression (91). In particular, Stats rely on their carboxy-terminal TAD to bind to co-activators. The TADs in Stat1, Stat2, Stat3, Stat5 and Stat6 are all known to interact with histone acetyltranferases (HATs) (90), and the p300/CBP HAT appears to be highly recruited to the Stat proteins (107). In addition to HATs, interactions with other proteins can occur at the same time to produce synergistic activation of gene expression. A variety of such interactions have been described. Stat3 has been reported to bind to c-jun, GR, and androgen receptor (108-110), Stat6 can bind to CCAAT/enhancer binding protein (C/EBP) (111), Stat5 can bind to GR and PR (112), and Stat1 can bind to Sp1 (113). Interactions between Stat dimers can also occur at special tandem binding sites on the DNA, allowing multiple dimers to bind, resulting in

maximal transcription activation. To date, there is no record of interactions between dimers of different Stat proteins (i.e. Stat3 and Stat5).

In addition to tyrosine phosphorylation, serine phosphorylation, arginine methylation and acetylation of stats has been reported, however details about these pathways are limited (91). The best described is a serine phosphorylation within the carboxy-terminus, which has been shown to enhance the transcriptional activity of most Stat proteins (114). Some co-activators are known to bind more readily when Stats are phosphorylated on this serine residue in addition to the tyrosine residue in the TAD (114). However, this does not hold true for all Stat proteins, since mutation of the conserved serine in Stat5 did not affect transcriptional activation of a PRL-responsive promoter in COS cells (115). The most simple explanation for this is that phosphorylation of the serine residue in Stat5 proteins may not affect transcription. Alternatively, the requirement for a phosphorylated serine could be promoter or cell type-specific. There is a great deal of speculation about the kinases responsible for serine phosphorylation of Stats, and several candidates within the p38, ERK, PKC, mTOR and JNK pathways have been implicated (114).

The length and intensity of a cytokine response is determined by the balance of activation and inactivation of the Stat pathway, and ultimately by the number of active Stat complexes in the nucleus. Stats are exported back to the cytoplasm in a process that appears to be mediated by the Crm1/Ran-GTPase (95), and this export depends on residues in coiled-coil domain and DBD (95, 116). Dephosphorylation of Stats in the nucleus initiates the nuclear export pathway. TC45 is one nuclear tyrosine phosphatase linked to the dephosphorylation of Stat1 and Stat3 (117). Based on studies using

staurosporine to inhibit tyrosine phosphorylation in fibroblasts, it takes approximately 20 minutes for an individual Stat1 molecule to go through the activation and deactivation cycle (118).

Besides dephosphorylation, negative regulation of Stat activity in the nucleus is controlled by at least two other mechanisms. The first is through the actions of a family of proteins called proteins that inhibit activated Stats (PIAS). These proteins interact only with tyrosine-phosphorylated Stats, and have been shown to block DNA binding of Stats, as well as inhibit their transcriptional activity by sumoylation (119). The second mechanism is via a set of naturally occurring truncated Stats which act as dominant negative forms of the proteins (91). Shortened forms Stat1, Stat3 and Stat5 have all been found (108, 120, 121). These proteins inhibit the Stat pathway because they contain a truncated carboxy-terminus that lacks a fully functional TAD. Thus, truncated Stats can compete with full length Stats for DNA binding and inhibit the activation of gene expression.

In addition to the factors that deactivate Stat proteins in the nucleus, negative regulators of the Jak/Stat pathway also exist in the cytoplasm. The supressors of cytokine signaling (SOCS) proteins are a family of molecules that act as negative regulators by binding to receptor and/or Jak catalytic sites to block further phosphorylation of Stat proteins. SOCS proteins can also induce the turnover of Jaks and some receptors via the proteasome degradation pathway by recruiting ubiquitin ligases to the activated receptor complex (122). To date, SOCS are the only known inducible inhibitors of the Jak/Stat pathway. The expression of SOCS mRNA is directly induced by Stat transcription factors, thus providing a negative feedback loop (123). A final mechanism of Stat

attenuation is via the SH2-containing phosphatase (SHP) proteins. These proteins are constitutively expressed, and can attenuate Stat signaling by dephosphorylating both Jaks and receptors.

Stat function: normal and disregulated

Stat proteins were first discovered during the analysis of interferon signaling pathways (124, 125). It is now known that Stats are activated by a large number of cytokines, and participate in many of the cellular processes, such as proliferation, differentiation, apoptosis, and cell survival, that are induced by cytokine, growth factor, and hormone signaling (126). One of the main physiological roles of Stat proteins is to regulate the immune response to infection. Stats 1, 2, 3, 4 and 6 have all been implicated in host defense, and the deletion of these genes in mice leads to a higher sensitivity to infections (91). Many different aspects of the immune response are regulated through the Stat pathway, including interleukin signaling, differentiation of T cells, and lymphocyte survival (91). Stats 3 and 5 have also been implicated in mammary gland development, and a detailed description of these functions is presented in a later section.

Signaling pathways that regulate proliferation and survival are frequently deregulated in cancer cells, and the Jak/Stat pathway is no exception. Increased expression of Stat1 has been observed in many human neoplasias (127). However, contradictory to this, Stat1 is also considered a potential tumor suppressor, since it promotes growth arrest and apoptosis (127). Thus, Stat1 may play multiple roles in the process of malignant transformation, and these roles are not yet clearly understood. In contrast, Stat3 and Stat5 are considered potential oncogenes, since they can induce

expression of c-Myc, cyclin D1, and bcl-xl, promoting cell-cycle progression, cellular transformation, and preventing apoptosis (126). Constitutively activated Stats have been observed in a number of human cancer cell lines and primary tumors (126). A persistently active form of Stat3 appears to be required for the survival of many head and neck and multiple myeloma cancer cells, since the introduction of a dominant-negative Stat3 into these cells often induces apoptosis (91). The activation of Stat3 in these cells has been shown to be the result of excess production of EGFR ligand, increased amounts of normal EGFR, or mutations that increase EGFR activity (128).

Stat5 expression in the mammary gland

Two members of the Stat family, Stat3 and Stat5, have been well documented as regulators of mammary gland development. While both proteins are critical for the normal function of the gland, they are believed to play opposing roles (129). Stat3 has been demonstrated to induce mammary involution by promoting apoptosis (130, 131), whereas Stat5 appears to inhibit this process (132). In addition, Stat5 is necessary for robust mammary development during pregnancy, and is vital for lactation. This review will focus on the functions of Stat5 in the murine mammary gland.

The Stat5 gene was originally cloned from lactating sheep mammary glands (133) and the protein was identified as a "mammary gland factor" (MGF) that is stimulated by PRL to initiate the expression of milk proteins (134). There are two closely related isoforms of Stat5, a and b, each encoded by a separate gene. In the mouse, the genes are located within a 110 kb stretch on chromosome 11 in a head to head orientation (135). Stat5a and Stat5b show 96% similarity at the amino acid level. They differ mainly within

the carboxy-terminus, where the last 8 amino acids of Stat5b are completely different from Stat5a, and Stat5a is 12 amino acids longer (136). When activated by phosphorylation, the two isoforms can homo- or hetero-dimerize. Stat5a and Stat5b carry out distinct functions *in vivo*. For example, Stat5b is selectively important for GH signaling in the liver (137, 138), while Stat5a is critical for the mammary gland development induced by PRL (138, 139). However, these differences may not be the result of differences in the proteins themselves, but rather due to the differential expression of each isotype. For example, Stat5b is the predominant isoform in the liver (140), while Stat5a is more highly expressed in mammary tissue (138).

The expression of Stat5a and Stat5b (collectively referred to as Stat5 from here on) is developmentally regulated in the mouse mammary gland. Northern analysis of whole mammary gland homogenates showed that Stat5 mRNA was only slightly detectable in the virgin and early pregnant gland, increased dramatically during pregnancy, and remained high in lactating glands (141). A similar expression pattern was found in the rat when mRNA levels of the Stat5a isoform were measured (142). Protein expression of both Stat5a and Stat5b isoforms was measured by Western blotting of whole mammary gland homogenates, and exhibited a developmental pattern similar to the mRNA. While total Stat5 protein levels increased during pregnancy and lactation, the phosphorylation of both isoforms increased even more significantly (143). Both the protein and phosphorylation levels of Stat5 dropped dramatically after weaning. One limitation of these studies is that they examined protein expression in the entire mammary gland, and the relative levels in the epithelium and stroma cannot be determined from the results. More recently, an immunohistochemical study reported that

phosphorylated Stat5 was present in adult virgin mouse and human epithelial cells (144), but other developmental stages were not examined.

Stat5 activation in the mammary gland can result from signaling through the GHR, PRLR or EGFR. PRL activates Stat5 only in epithelial cells due to the absence of PRLR in the stroma, while GHR and EGFR activate Stat5 primarily in the stromal compartment (61). Pituitary factors are the predominant activators of Stat5 in the non-pregnant gland, as demonstrated by the lack of phospho-Stat5 staining following hypophysectomy (144). The finding that PRLR-/- mice exhibit an 80% reduction in the level of phosphorylated Stat5 in the mammary gland indicated that PRL is the primary pituitary factor that induces Stat5 activation (144). Jak2 was shown to mediate the activation of Stat5 by PRLR in response to PRL treatment (145), and in agreement with this, PRLR-/-, Jak2-/- and Stat5-/- mammary glands all exhibit similar defects alveolar development (55, 146, 147).

Stat5 function in the mammary gland

The use of knockout models has been critical to understanding the physiology of Stat5 in the mammary gland. Two approaches have been taken to produce Stat5 null mice. The first knockout models were made by the germline deletion of either the entire 110kb Stat5 locus, or of the Stat5a and Stat5b genes independently (referred to as Stat5-/-, Stat5a-/- and Stat5b-/- mice from here on) (138, 139, 147). Additionally, two mammary specific knockouts of the entire Stat5 locus were produced to avoid the reproductive defects seen in Stat5-/- mice, and to examine defects during later development (148). The conditional knockouts were generated by creating a mouse line which contained the

Stat5a and Stat5b genes flanked by *loxP* recombination sites. These mice were then bred with two transgenic strains, each of which contained the gene for *cre* recombinase under the control of a mammary specific promoter. Expression of *cre* recombinase in one mouse line was controlled by the MMTV promoter, which is active in the mammary gland epithelium throughout development. The second mouse line expressed the *cre* recombinase transgene under the control of the WAP promoter, which is activated by Stat5 in the late pregnant and lactating mammary gland. Thus, in the second system, Stat5 was deleted only in cells that had already become fully differentiated.

Germline deletion of both Stat5 isoforms resulted in incomplete alveolar development, and a complete absence of lactation (138, 147), which was similar to the mammary phenotypes seen in Jak2-/- (146) and PRLR-/- mice. When only the Stat5a isoform was deleted, mammary development was comparable to the total Stat5 knockout (138, 139). In contrast, deletion of Stat5b was reported have either no effect or an extremely blunted effect on mammary development when compared to Stat5a knockouts (138, 139). This difference is likely due to the disparity in expression levels between the Stat isoforms in the mammary gland (138). In concordance with this model, Stat5b was able to compensate for the loss of Stat5a after multiple pregnancies in Stat5a-/- mice, and an increase in Stat5b levels was observed in these mice (149).

Another important phenotype of Stat5-/- mice to note is their infertility. This defect was determined to be the result of non-functioning corpora lutea, the PRL stimulated exocrine glands in the ovary that secrete P (138). PRL has a critical role in ovarian function, as indicated by the fact that both PRL-/- mice (150) and PRLR-/- mice are infertile (53), as well as the role of PRL in activating Stat5. It is interesting to note

that both Stat5a -/- and Stat5b -/- mice are fertile, indicating the functional redundancy of the Stat5 isoforms in the ovary. However, it is not known if the corpora lutea are maximally functional in these animals. To circumvent the problem of infertility in Stat5 knockout mice, mammary gland transplantation was used to examine mammogenesis during pregnancy.

Mammary glands comprised of Stat5-/- epithelium grown in wild-type stroma exhibited a lack of functional alveoli at parturition (147). Further investigation determined that this phenotype was associated with a defect in proliferation in response to E+P treatment. Wild-type mice were transplanted with Stat5-/- epithelium, and mammary glands were allowed to develop for 6 weeks. The mice were then treated with an acute 2 day dose of E + P to mimic early pregnancy, and proliferation was assessed by the percentage of cells that incorporated BrdU during a short labeling period prior to sacrifice. The results showed that there were approximately 50% fewer 5-Bromo-2'-Deoxyuridine (BrdU) positive cells in Stat5-/- epithelium compared to wild-type glands (147). Similarly, MMTV-cre recombinase Stat5-/- mice showed an 80% decrease in mammary epithelial cells positive for the proliferation marker, phosphorylated histone 3 (H3P) in response to the same 2 day hormone treatment (148). Together, these findings demonstrate that a major function of Stat5 in the mammary gland is to mediate the proliferation of epithelial cells in response to ovarian hormones.

Stat5 has also been described as a regulator of mammary cell differentiation, and this role appears to be especially critical in the pregnant and lactating gland. Evidence for this function comes from the observation that epithelial cells in Stat5-/- mammary glands do not express markers of fully differentiated cells during pregnancy, and instead

maintain virgin-like features (147). The expression of the Na-K-Cl cotransporter (NKCC1) is normally present in the virgin mammary epithelium, but at much lower levels in alveoli during pregnancy. When the alveolar-like structures in pregnant Stat5-/- mammary glands were examined, expression of NKCC1 was maintained at the level seen in virgin glands. Further investigation found that the sodium phosphate cotransporter Npt2b, a protein that is normally expressed at high levels in the lactating gland, was not detectable in Stat5-/- mammary cells at parturition. In agreement with these findings, expression of markers of fully differentiated secretory cells, WAP, β-casein, and α-lactalbumin, was decreased in Stat5a-/- glands at parturition.

Genetic disruption of Stat5 in the mammary gland has also uncovered a role for the protein in regulating cell survival. The loss of Stat5 late in pregnancy in WAP-*cre* recombinase Stat5-/- mice resulted in premature mammary cell death via apoptosis. When expression of the *cre* recombinase protein was examined during pregnancy, the number of positive cells continually decreased, and very few expressing cells remained by day 18.5 of pregnancy. This indicated that cells producing *cre* recombinase, and thus lacking Stat5 expression, were not able to survive in the pregnant gland. Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) revealed that ~5 times more apoptotic cells were present in the WAP-*cre*-recombinase Stat5-/- mammary glands at day 15.5 of pregnancy compared to control glands. In summary, the results obtained from the Stat5 knockout studies have demonstrated that Stat5 controls the formation of functionally differentiated alveoli during pregnancy through the regulation of proliferation, differentiation and survival of mammary epithelial cells.

Stat5 regulated genes

A number of genes are transcriptionally regulated by Stat5 and, as predicted by its roles in the mammary gland and other tissues, many of these genes contribute to cell proliferation, survival, and differentiation. Gene array experiments have contributed to defining which genes are induced by Stat5 in the mammary gland. The induced dimerization of Stat5-gyrase fusion proteins in KIM2 mammary epithelial cells elicited the upregulation of numerous genes (132). Of the most highly induced genes, many encoded epithelial differentiation markers and anti-apoptotic proteins, such as α-casein and prosaposin, respectively.

Many of the genes involved in differentiation that are directly induced by Stat5 in the mammary gland encode milk constituents such as WAP, β-casein and α-lactalbumin. However, Stat5 can also induce the expression of other transcription factors, which in turn can regulate expression of milk genes. Expression of Ets1 was found to be directly regulated by Stat5 (132), and Ets1 has been shown to induce the WAP gene (151). Elf5, another member of the Ets family of transcription factors, is hypothesized to be induced by Stat5, and it also regulates milk production in the mammary gland (152).

Stat5 can also activate transcription of several genes implicated in proliferation and survival. Expression of RANKL is induced by PRLR signaling through the activation of Stat5 in mammary epithelial cells (153). Multiple pathways may be regulated by RANKL in mammary cells, and the exact mechanisms by which it induces proliferation are not yet clear. Stat5 can also activate transcription of the cyclin D1 gene directly at GAS elements within the promoter, and Stat5 has been observed to bind to the cyclin D1 gene promoter in the MCF-7 and HC-11 mammary epithelial cell lines in

response to hypoxic conditions (154). Finally, expression of the anti-apoptotic protein Bcl-xl has been shown to be induced by Stat5 in several systems, but it is not known whether this occurs in mammary epithelial cells (155).

Stat5 and breast cancer

In addition to its role in normal mammary gland development, Stat5 has also been implicated in mammary oncogenesis. Transgenic animals that express either wild-type or a constitutively active form of Stat5 in the mammary gland develop mammary tumors at an incidence rate of up to 22% within 8-12 months (156). In addition, the deletion of Stat5 impairs tumor development in mice with mammary specific over-expression of TGF-α or SV40 large-T antigen (157, 158). Finally, PRL is a reported tumor promoter in rodents (159), and this may be via the activation of Stat5.

Stat5 may also play a role in human breast cancer as well, although the data are somewhat paradoxical. An early study examined Stat5 activity in extracts from a small number of invasive carcinomas, benign lesions and normal tissue by electrophoretic mobility shift assay (EMSA) (160). The majority of invasive carcinomas exhibited increased Stat5 activity compared to the other two sample types, suggesting that Stat5 may aid in the progression of tumors. Although this study presented evidence for changes in Stat5 activity in breast cancer, the conclusions that could be drawn were limited by a lack of normalization to total Stat5 protein levels, and a small sample size.

Data obtained in a number of more recent studies using larger sample sizes somewhat oppose the early findings. When nuclear-localized, tyrosine phosphorylated Stat5 was scored in tumor samples from more than 1000 patients, tumors with active

Stat5 corresponded to a reduced risk of tumor recurrence and patient morbidity (161). The prognosis based on Stat5 activity was independent of many other predictors of survival including ER and PR status, Her2/neu status, tumor size, and histological grade. The link between Stat5 activity and patient survival may be due to its ability to suppress tumor invasiveness. Activation of Stat5 via PRL in both T-47D and ZR-75-1 breast cancer cell lines leads to increased levels of E-cadherin, an adhesion molecule known to suppress cell invasion (162).

Although it seems somewhat contradictory, Stat5 appears to play a positive role in tumor formation and an inhibitory role in tumor progression. The ability of Stat5 to induce proliferation may explain why constitutively active forms of the protein can lead to tumor formation. Additionally, increased Stat5 activity could lead to a larger pool of epithelial cells that can become transformed. However, once tumors have formed, the presence of active Stat5 could prohibit metastasis through inducing differentiation of tumor cells, potentially explaining the increase in patient survival.

Conclusion

Mammary gland development is a dynamic process that requires a network of several signaling pathways. Numerous studies have established the signaling molecule, Stat5a as one of the critical regulators of pregnancy-induced mammary gland development. Since its discovery as a key mediator of PRL signaling, considerable progress has been made in defining the mechanisms leading to Stat5a activity. However, several aspects of Stat5a in the mammary gland are still relatively unknown. The underlying mechanisms that regulate Stat5a expression in the mammary gland are not

known. In addition, the function of Stat5a outside of pregnancy has not been thoroughly explored. Finally, the mechanisms by which Stat5a controls proliferation in the mammary gland have not been elucidated. The research presented in this dissertation addresses these aspects, and provides new insight into both the regulation and function of Stat5a in the mouse mammary gland.

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CHAPTER TWO

ESTROGEN AND PROGESTERONE ARE CRITICAL REGULATORS OF STAT5A EXPRESSION IN THE MOUSE MAMMARY GLAND

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ABSTRACT

Signal transducer and activator of transcription (Stat)5a is a well-established regulator of mammary gland development. Several pathways for activating Stat5a have been identified, but little is known about the mechanisms that regulate its expression in this tissue. In this report, we used immunofluorescent staining to examine Stat5a expression in mammary epithelial cells during normal development and in response to treatment with the ovarian hormones estrogen (E) and progesterone (P). Stat5a was present at very low levels in the pre-pubertal gland, and was highly induced in a subset of luminal epithelial cells during puberty. The percentage of positive cells increased in adult virgin, pregnant, and lactating animals, dropped dramatically during involution and then increased again post-weaning. Ovariectomy ablated Stat5a expression in virgin animals, and treatment with both E and P was necessary to restore it. Double labeling experiments in animals treated with E+P for three days demonstrated that Stat5a was localized exclusively to cells containing both estrogen and progesterone receptors. Together, these results identify a novel role for E and P in inducing Stat5a expression in the virgin mammary gland, and suggest that these hormones act at the cellular level through their cognate receptors.

INTRODUCTION

Signal transducer and activator of transcription (Stat)5 plays an important role in mammary gland development. Two isoforms of Stat5, a and b, are produced by separate genes (1), and act as signaling mediators involved in numerous cellular functions including proliferation, differentiation and survival (2-4). They are members of the Stat family of proteins, which are latent transcription factors that localize to the cytoplasm until activated by a variety of cytokines, growth factors and hormones (5-7). Binding of these ligands to their cognate receptors activates either an intrinsic receptor kinase domain or an associated Jak kinase, which then recruits and phosphorylates Stat proteins (8, 9). Phosphorylated Stat proteins translocate to the nucleus where they bind DNA and activate responsive genes.

The majority of studies of Stat5 in the mammary gland have focused on its role in pregnancy and lactation. When both a and b isoforms were deleted, Stat5-/- mice exhibited reduced alveolar expansion during pregnancy and a lactational defect (10). A similar phenotype was seen when only the Stat5a isoform was deleted, but Stat5b deficient mice had a much less severe mammary gland defect (10-12). This indicates a specific requirement for Stat5a during mammary gland development, which is consistent with the fact that it is the predominant isoform expressed in this tissue (10).

In terms of specific functions, Stat5a has been reported to play important roles in mammary cell differentiation, proliferation and survival. In the final stages of mammary cell differentiation, it activates expression of genes encoding milk constituents such as α-lactalbumin, β-casein and whey acidic protein (WAP) (10, 13). Further supporting a role

for Stat5a in differentiation, recent studies have demonstrated that after parturition, Stat5-/- epithelial cells lack a specific marker of alveolar cells (Npt2b), while retaining a marker of virgin-like ductal cells (NKCC1) (14, 15). Stat5a may also play a role in the proliferative response to estrogen (E) and progesterone (P) during pregnancy, since BrdU incorporation was significantly decreased in Stat5-/- mouse mammary epithelial cells in response to E+P treatment (14, 15). Finally, conditionally deleting Stat5 (a and b) during pregnancy induced premature cell death, indicating that it is critical for cell survival at this developmental stage (14).

Developmental studies using Northern and Western blotting of whole murine mammary gland homogenates demonstrated that Stat5a is present in both the immature and mature virgin, increases during pregnancy, and reaches a maximum level during late pregnancy and lactation (16, 17). However, expression in the stromal and epithelial compartments could not be discriminated using these approaches, and the increase in Stat5a might therefore reflect increased epithelial cell number rather than increased expression per epithelial cell. In addition, Western blotting does not permit one to localize expression to specific epithelial structures such as ducts, end buds and alveoli. A more recent study used immunohistochemistry to examine total Stat5 expression in mouse mammary epithelium. It was found that Stat5 was expressed in the adult virgin as well as the pregnant gland, but immature animals were not examined (18). Surprisingly, the majority of Stat5 was activated, even in virgin animals. In the mammary gland, Stat5 can be activated by growth hormone, EGF, or prolactin (PRL) (19, 20). In lactating animals, Stat5a induces expression of milk protein genes, largely in response to PRL, and Stat5a activation in virgin animals also seems to be predominantly due to PRL (18).

Although the pathways leading to Stat5a activation have been extensively studied, little information is available regarding the regulation of its expression in mammary cells. Progestin treatment was reported to induce Stat5a transcription in the human breast cancer cell line, T47D. There are two forms of the progesterone receptor (PR), PRA and PRB (21), and Stat5a induction occurred only in cells expressing the PRB isoform (22). However, the factors regulating Stat5a expression in the normal mammary gland have not been identified. In the current work, we have utilized immunofluorescence to study Stat5a expression in mouse mammary epithelial cells during normal development and in response to hormone treatments. The results demonstrate that Stat5a expression increases dramatically at puberty and after ovariectomy in response to the ovarian hormones E+P. Furthermore, Stat5a extensively co-localizes with both estrogen and progesterone receptors (ER and PR), suggesting that it is a target of these receptors and may mediate some of the effects of E and P in the mammary gland.

MATERIALS AND METHODS

Animal Experiments:

18 week old virgin, female BALB/c mice from our own colony were bilaterally ovariectomized one week prior to hormone treatment. Animals were injected subcutaneously in the dorsum of the neck with saline, 17β-estradiol (Sigma, Saint Louis, MO) (1 μg), progesterone (Sigma) (1 mg), or the combination of both hormones every 24 h for either 3 or 10 consecutive days. Prolactin (Sigma) was injected subcutaneously

every 12 h (1 μg/g body weight per day). For bromocriptine (BC) experiments, 0.5 mg of BC (Sigma) was suspended in 0.1 ml sesame oil (Sigma) and administered subcutaneously every 24 h for 3 days. This amount of BC is reported to be adequate for a sustained suppression of serum prolactin levels for 24 h (23). Animals were injected intraperitoneally with 70 μg/g body weight of 5-bromo-2-deoxyuridine (BrdU) (Sigma) two h before they were euthanized. All animal experimentation was conducted in accordance with accepted standards of humane animal care and approved by the All University Committee on Animal Use and Care at Michigan State University.

Antibodies:

Rabbit polyclonal α-Stat5a (cat# sc-1081), goat polyclonal α-phospho Stat5 (cat# sc-11761) and rabbit polyclonal α-WAP (cat# sc-25526) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal α-Stat5a (cat# 611834) antibody was obtained from BD Biosciences (San Jose, CA). Goat polyclonal α-RANKL (AF462) antibody was obtained from R&D Systems (Minneapolis, MN). Mouse monoclonal α-smooth muscle actin antibody (clone 14A) was from Sigma. Mouse monoclonal α-human ERα antibody (NCL-ER-6F11) was obtained from Novocastra Laboratories (Newcastle, UK). Rabbit polyclonal α-PR, which detects only PRA (cat# A0098) was obtained from Dako (Carpinteria, CA). Rabbit polyclonal α-PRB was produced commercially using a peptide corresponding to 15 amino acids unique to mouse progesterone receptor B by Affinity Bioreagents (Golden, CO) and has been verified to detect only PRB (24). Mouse monoclonal α-BrdU antibody was provided in a kit from

Amersham Biosciences (Piscataway, NJ). All secondary antibodies were conjugated to Alexa fluor dyes and were obtained from Invitrogen (Carlsbad, CA).

Immunohistochemistry:

Inguinal mammary glands were fixed in 10% phosphate-buffered formalin overnight at 4 C, dehydrated, cleared and embedded in paraffin. Tissue was sectioned to 5 μm, mounted on 3-aminopropyl triethoxysilane-coated coverslips, and allowed to dry for 24 h at room temperature. The tissue was then dewaxed and rehydrated through a descending gradient of ethanol. Sections were autoclaved (121 C at 15 psi) for 20 min in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. Specific blocking and antibody incubation protocols are detailed below. Each incubation step was followed by two 5 min washes in phosphate buffered saline (PBS).

For Stat5a labeling, sections were blocked for 30 min in 2% bovine serum albumin in PBS, pH 7.3 (2% PBSA). Samples were first incubated with rabbit α-Stat5a antibody (in 2% PBSA, overnight at 4 C), then incubated with goat α-rabbit antibody conjugated to Alexa 488 (green) (in PBS, 30 min). For double labeling experiments, sections were first blocked with goat α-mouse IgG Fab fragments (Jackson ImmunoResearch Laboratories, West Grove, PA) [1:100 1% PBSA, 1 h], then blocked with normal goat serum (NGS) (Vector Laboratories, Burlingame, CA) (1:1 in PBS, 30 min). The tissue was incubated with either mouse α-BrdU for 1 h, or mouse α-SMA (in PBS), α-ERα (in PBS-0.5% Triton X-100) or α-Stat5a antibody (in PBS) overnight at 4 C. This was followed by incubation with goat α-mouse Alexa 488 for Stat5a-PRA

double labeling or Alexa 546 (red) for all other staining (in PBS, 30 min). Samples were blocked with 2% PBSA for 30 min, incubated with one of the following: rabbit α-Stat5a, α-WAP, α-PRA or α-PRB antibody in 2% PBSA overnight at 4 C. This was followed by incubation with goat α-rabbit Alexa 546 for Stat5a-PRA labeling or Alexa 488 for all other staining (in PBS, 30 min). For Stat5a-RANKL double staining: sections were incubated with normal rabbit serum (1:1 in PBS, 30 min), α-RANKL antibody (in PBS 0.5% Triton X-100, overnight at 4 C), rabbit α-goat Alexa 488 (30 min), goat α-mouse IgG Fab (1:100 in 1% PBSA, 1 h), NGS (1:1 in PBS, 30 min), mouse α-Stat5a (overnight at 4 C), and goat α-mouse Alexa 546 (30 min). For Stat5a and SMA, WAP, RANKL, ERα, PRA or BrdU staining, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) and pictures were taken on a Nikon Eclipse fluorescent microscope. Intensity measurements were made using MetaMorph 6 Software. For Stat5a and PRB double staining, nuclei were counterstained using TOPRO-3 iodide (Molecular Probes), and visualized using a Pascal laser scanning confocal microscope (Zeiss). For Stat5a visualization following BC treatment, nuclei were counterstained using TOPRO-3 iodide and visualized using a FluoView laser scanning confocal microscope (Olympus).

To detect phosphorylated Stat5, antigen retrieval was accomplished as previously described (20). After blocking in 10% normal rabbit serum (NRS) (Vector Laboratories) in PBS for 30 min, the sections were incubated overnight a 4 C with goat α-phospho-Stat5 antibody (in 10% NRS in PBS). The tissue was then incubated with rabbit α-goat Alexa 488 antibody (in PBS, 30 min). Nuclei were stained with DAPI, and samples were visualized using a Nikon Eclipse microscope and MetaMorph software.

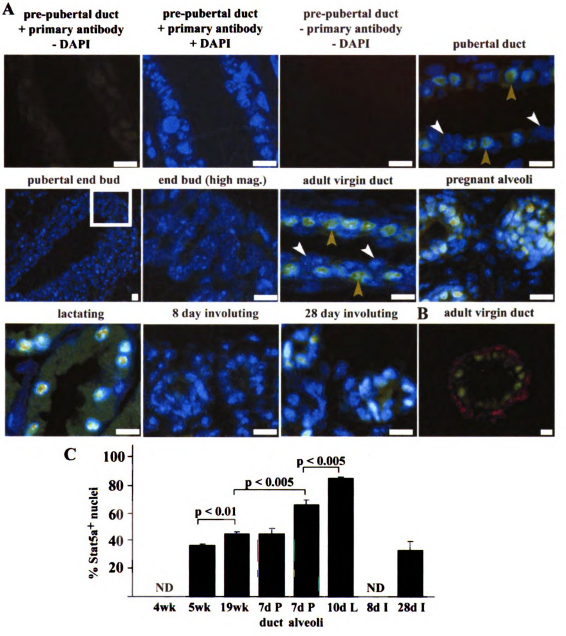
RESULTS

Immunofluorescent localization and quantitation of Stat5a in epithelial cells during mammary gland development

To examine Stat5a expression during mammary gland development, the level and pattern of expression was examined by immunofluorescence in mammary tissue from prepubertal (4 wk), pubertal (5 wk), adult (19 wk), pregnant (7 day), lactating (10 day), and involuting (8 and 28 day) animals (Fig. 2.1A). In 4 wk old animals, a diffuse signal that was slightly above the secondary antibody alone control was observed. By 5 weeks of age, intense staining was seen in a subpopulation of luminal epithelial cells that were present throughout the ducts in a punctate pattern. An exception to this punctuate pattern was noted in end buds, where only diffuse light staining was present. Most of the intense staining in ductal cells was nuclear, suggesting that the protein is activated even at this early age. In mature (19 wk) virgin animals, where the ducts have reached the limit of the fat pad and end buds are no longer present, the punctate distribution of positive cells persisted, and the staining remained predominantly nuclear.

Since Stat5a is required for lobuloalveolar development and lactation, its expression was also examined in the pregnant, lactating and involuting mammary gland (Fig. 2.1A). Intense nuclear Stat5a staining was observed in both ductal and alveolar cells in the pregnant mammary gland, and an increase in cytoplasmic staining was also observed in the $Stat5a^{+}$ alveolar cells. In sections from lactating animals, the vast majority of cells exhibited both intense nuclear and cytoplasmic Stat5a staining.

Figure 2.1. Immunofluorescent staining of Stat5a during mouse mammary gland development. A. Sections from 4 wk old pre-pubertal, 5 wk old pubertal, 19 wk old mature adult, 7 day pregnant, 10 day lactating, and 8 and 28 day involuting mammary glands were stained with α-Stat5a antibody (green) and nuclei were labeled with DAPI (blue) as described in Materials and Methods. Control sections from 4 wk old glands without primary antibody are also shown. Green arrowheads indicate Stat5a positive cells and white arrowheads indicate Stat5a negative cells. B. Mammary glands from mature (19 wk old) mice were stained for smooth muscle actin (red) and Stat5a (green). Scale bar for all images = 5μm. C. Quantitation of nuclear Stat5a expression at different stages of mouse mammary gland development. Immunofluorescent staining using an α-Stat5a antibody was carried out as described in (A) on tissue sections from 4-, 5-, or 19-week old virgin, 7 day pregnant (P), 10 day lactating (L), 8 or 28 day involuting (I) mice. The values represent the mean ± SEM from three animals per group, with a minimum of 1000 cells/mouse analyzed, and significance was determined by Student's *t* test. No nuclear staining was detected (ND) at 4 weeks of age or 8 days of involution. Images in this dissertation are presented in color.


A
pre-pubertal duct
+ primary antibody
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pre-pubertal duct
+ primary antibody
+ DAPI
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pubertal duct
pubertal end bud
end bud (high mag.)
adult virgin duct
pregnant alveoli
lactating
8 day involuting
28 day involuting
B
adult virgin duct
C
% Stat5a+ nuclei
100
80
60
40
20
p < 0.005
p < 0.005
p < 0.01
ND
ND
4wk
5wk
19wk
7d P
7d P
10d L
8d I
28d I
duct
alveoli

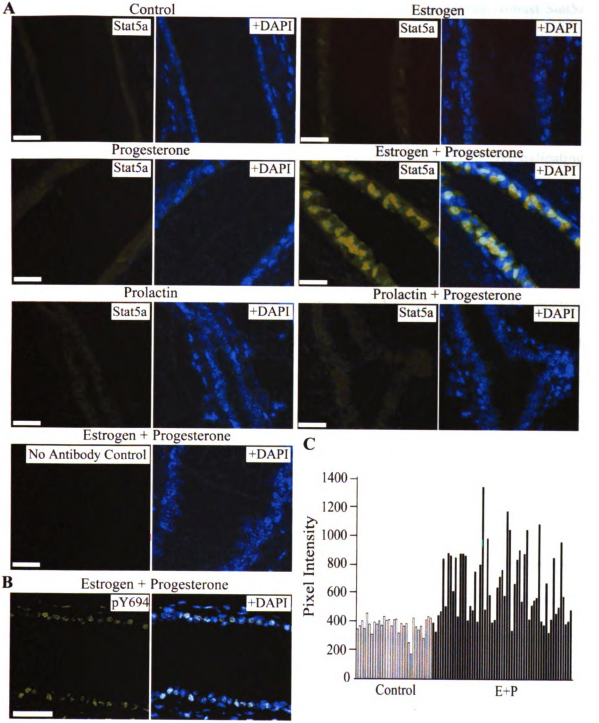
Expression was dramatically decreased in the 8 day post-lactational involuting gland, where staining was less intense than in the virgin gland and resembled that seen in the pre-pubertal gland. However, Stat5a staining was restored by 28 days after weaning, and approached the level seen in the adult virgin gland. Double staining for Stat5a and the myoepithelial cell marker, smooth muscle actin, demonstrated that Stat5a expression was limited to luminal epithelial cells (Fig. 2.1B).

Quantitation of the results shown in figure 2.1A revealed that 37% of luminal epithelial cells had intense nuclear Stat5a staining in 5 wk old animals, and this increased to 46% in 19 wk old mice (Fig. 2.1C). Stat5a was expressed at similar levels in ducts in the pregnant gland, where 45% of luminal epithelial cells were positive. Expression was higher in the alveoli, where 67% of cells were positive. Expression was highest during lactation, with 87% of cells staining positive. Stat5a staining was non-detectable at day 8 of involution, but was present 28 days after weaning, where 34% of cells were positive.

Regulation of Stat5a expression by ovarian hormones

The appearance of Stat5a between 4 and 5 weeks of age, when ovarian cycles typically begin, and the further increase during pregnancy, suggested that its expression might be regulated by the ovarian hormones E and P. To test this possibility, 18 week old mice were ovariectomized and allowed to recover for 1 week. They were then injected with E, P, E+P, or vehicle control once per day for 3 days, and their mammary glands were examined for Stat5a expression. After ovariectomy, control-treated animals had weak Stat5a staining (Fig. 2.2A), similar to that seen in the 4 wk old, intact pre-

Figure 2.2. Stat5a expression in hormone treated mice. A. 18 wk old virgin mice were ovariectomized, then treated with vehicle control, E, P, PRL, E+P or PRL+P for 3 days as described in Materials and Methods. Representative photomicrographs of Stat5a staining are shown. B. 3 day E+P treated mammary glands were stained with an α-pY694 Stat5 antibody. All images show Stat5a staining (green) in the left panel and the merged image with DAPI- stained nuclei in the right panel. Scale bar for all images = 20 μm. C. Quantitation of Stat5a staining intensity in E+P treated mice. The total pixel intensity of every cell (nucleus + cytoplasm) was measured from a single representative structure from the 3 day control (white bars) and E+P treated (black bars) mice (each bar = 1 cell). Images in this dissertation are presented in color.


A
Control
Stat5a
+DAPI
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Stat5a
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Estrogen + Progesterone
No Antibody Control
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B
Estrogen + Progesterone
pY694
+DAPI
C
Pixel Intensity
1400
1200
1000
800
600
400
200
0
Control
E+P

pubertal gland (see Fig. 2.1A). Ovariectomized animals treated with P alone were indistinguishable from the control-treated mice. Estrogen treated animals had a slight increase in nuclear Stat5a staining in a few cells (Fig. 2.2A). In contrast, robust Stat5a expression was observed in both the nucleus and cytoplasm in E+P treated mice, with 41±1% of luminal epithelial cells being positive (Fig. 2.2A). Overall, both the percentage and distribution of Stat5a$^+$ cells in the 3 day E+P treated animals were similar to that observed in the mammary glands of intact, mature mice (see Fig. 2.1A), indicating that expression of Stat5a in the mammary gland is largely dependent on ovarian hormones.

Nevalainen et al. previously used phospho-specific antibodies to demonstrate that Stat5 is activated in adult virgin mouse mammary epithelial cells (18), and the nuclear localization of Stat5a in the glands from E+P treated animals (Fig. 2.2A) suggested that Stat5a is also phosphorylated and activated under these conditions. To determine if this was the case, immunofluorescent staining was carried out with an antibody that detects both a and b Stat5 isoforms phosphorylated on tyrosine 694 (pY694) (Fig. 2.2B). When scored, 47±7% of luminal epithelial cells from E+P treated animals were positive for pY694, similar to the percentage of cells showing intense nuclear Stat5a staining. Since the Stat5a observed after E+P treatment was predominantly nuclear, we considered the possibility that some or all of the apparent increase in expression was actually due to translocation of the protein into the nucleus. To address this issue, the staining intensities of individual cells (cytoplasm + nucleus) in representative structures from the 3 day control and E+P treated mice were quantitated and compared. Cells with a low level of staining in the E+P gland had similar intensities to the cells in the control gland (Fig.

2.2A and C). However, cells with bright nuclear staining in the E+P gland had approximately twice the intensity of the other cells (Fig. 2.2A and C). This increase in intensity demonstrates that total cellular Stat5a protein levels are increased in a subset of luminal epithelial cells in response to E+P treatment.

PRL treatment increased the amount of activated Stat5 in hypophysectomized animals (18), and E can induce PRL secretion (25, 26). One potential role of E in these experiments could therefore be to induce PRL, which would then lead to Stat5a nuclear accumulation. To examine the possibility that PRL might increase expression as well as activation of Stat5a, ovariectomized virgin animals were treated for 3 days with PRL or PRL+P. Neither treatment increased Stat5a expression significantly compared to the control (Fig. 2.2A), indicating that E is not increasing Stat5a expression via PRL. However, E may contribute to Stat5a activation and nuclear localization via PRL. The results shown in Figure 2.2 demonstrated that E+P treatment increased Stat5a expression in the mammary epithelium, and that the protein was phosphorylated and nuclear. Since PRL is the major activator of Stat5a in the adult virgin gland (18), this suggested that E+P treatment in the absence of PRL would lead to increased protein without nuclear localization. To test this hypothesis, bromocriptine (BC) was utilized to inhibit the secretion of PRL from the pituitary gland. Ovariectomized mice were treated with E+P for 3 days as described above, and were additionally given BC or vehicle control. Mammary glands were examined by confocal microscopy to assess the cellular localization of Stat5a. As expected, strong nuclear Stat5a staining was observed in the tissue from E+P+vehicle treated mice (Fig. 2.3). In contrast, mammary epithelial cells from E+P+BC treated mice had predominantly cytoplasmic Stat5a staining (Fig. 2.3).

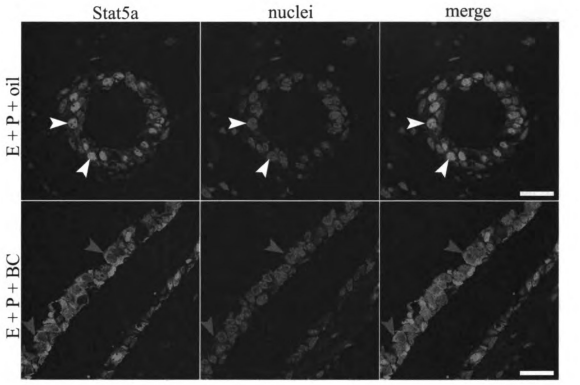


Figure 2.3. Cellular localization of Stat5a in bromocriptine treated mice. 18 wk old virgin mice were ovariectomized, and treated with E+P + vehicle control (oil) or E+P + bromocriptine (BC) for 3 days. Staining for Stat5a (green) and nuclei (red) was visualized with an Olympus laser scanning confocal microscope. White arrowheads indicate nuclear Stat5a staining and red arrowheads indicate cytoplasmic Stat5a staining. Scale bar = 20 μm. Images in this dissertation are presented in color.

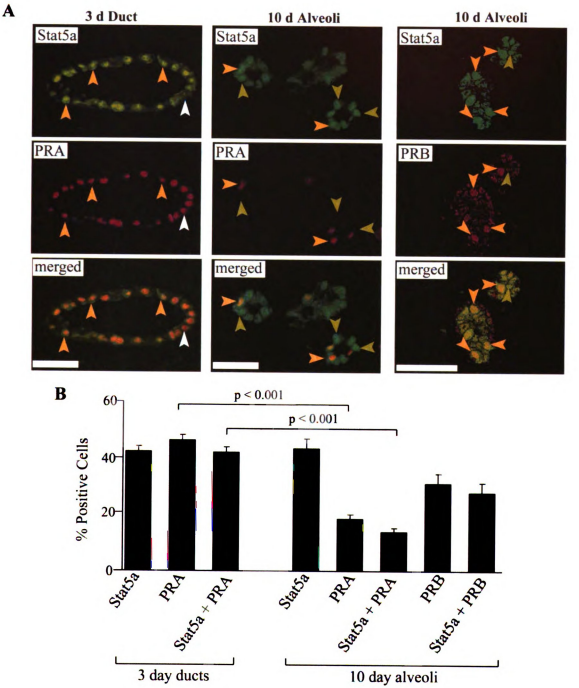
Based on these results, we propose a model where E+P induces the expression of Stat5a, which can subsequently be activated by PRL, leading to its nuclear localization.

Colocalization of Stat5a with estrogen and progesterone receptors

The induction of Stat5a expression by E+P suggested that the gene might be a target of the ER and/or PR. Previous studies in T47D human breast cancer cells have indicated that transcription of the Stat5a gene is induced by progestin in cells expressing PRB, but not PRA (22). However, PRA is the dominant isoform observed in virgin mice, where it is present in ductal epithelial cells in a punctate pattern that is reminiscent of that seen with Stat5a (27). In contrast, PRB is not detectable in adult virgin mice, increases during pregnancy, and is predominately localized in alveoli. To determine if Stat5a colocalizes with PRA or PRB in the mouse mammary gland, samples from the 3 and 10 day E+P treated animals were examined by double indirect immunofluorescent staining using Stat5a and PR-isoform specific antibodies. After 3 days of E+P treatment, only ducts were scored since no alveoli were present. In these ductal structures, 42% of luminal cells were Stat5a$^+$, 46% were PRA$^+$ and 42% were both Stat5a and PRA positive (Fig. 2.4). Thus, after 3 days of E+P treatment, virtually all cells expressing Stat5a contained PRA, but there was a small subset (~9%) of PRA$^+$ cells that did not express Stat5a.

We previously observed that PRB is absent from ducts and is only detectable in alveoli during pregnancy (27) or after 5 or more days of E+P treatment (28). To determine if Stat5a colocalizes with PRB, we examined Stat5a/PR colocalization in

Figure 2.4. Colocalization of Stat5a with PRA or PRB. A. Staining for Stat5a and PRA was carried out on sections from 3 and 10 day E+P treated ovariectomized mice, and was visualized with a Nikon Eclipse fluorescent microscope. Staining for Stat5a and PRB was carried out on sections from 10 day E+P treated mice only, and was visualized with a Pascal laser scanning confocal microscope. Yellow arrowheads indicate Stat5a and PRA or Stat5a and PRB colocalization (yellow/orange nuclei). White arrowhead indicates Stat5a negative, PRA positive cell (red nucleus). Green arrowheads indicate Stat5a positive, PRA or PRB negative cells (green nuclei). Scale bar = 20 μm. B. Quantitation of Stat5a and PR colocalization in 3 and 10 day E+P treated animals. The values represent the mean ± SEM from three animals per group, with a minimum of 800 cells/mouse analyzed. The percentage of PRA positive cells and Stat5a/PRA positive cells were both significantly lower in 10 day E+P alveolar structures than 3 day E+P ducts ($p < 0.001$) as determined by Student's *t* test. Images in this dissertation are presented in color.


A
3 d Duct
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% Positive Cells
60
40
20
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Stat5a
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Stat5a + PRB
3 day ducts
10 day alveoli

alveolar structures after 10 days of E+P treatment. As expected, PRB was not detected in ducts (data not shown), but was present in 31% of alveolar cells (Fig. 2.4). PRA was present in 18% of alveolar cells, compared to 47% of ductal cells from 3 day treated animals. When Stat5a expression was quantitated, 43% of alveolar cells were positive, which is similar to the percentage seen in the 3 day treated samples. Scoring for both Stat5a and PRA or PRB revealed that 35% of Stat5a expressing cells were PRA^+, and 65% were PRB^+ (Fig. 2.4). Since PRA and PRB are rarely expressed in the same cells in the mouse mammary gland (27), we infer from these data that virtually all $Stat5a^+$ alveolar cells are also PR^+, with roughly one third containing PRA and the other two thirds containing PRB.

Since both E and P were required to induce Stat5a expression in ovariectomized animals, we examined whether ERα and Stat5a colocalize in ductal epithelial cells from 3 day E+P treated mammary glands. Double staining revealed that the two proteins were highly colocalized, with 43% of cells being $Stat5a^+$, 45% $ER\alpha^+$, and 42% both Stat5a and ERα positive (Fig. 2.5). Thus, virtually all $Stat5a^+$ cells in ducts contain ERα. Since PRA also colocalizes with Stat5a in ducts, this suggests that a sub-population of ductal cells contain ERα, PRA and Stat5a.

Colocalization of Stat5a and BrdU

Mouse mammary glands lacking both Stat5a and b (Stat5-/-) display a significant reduction in epithelial cell proliferation in response to E+P treatment compared to wild-type glands, suggesting that Stat5 is a positive regulator of proliferation in response to

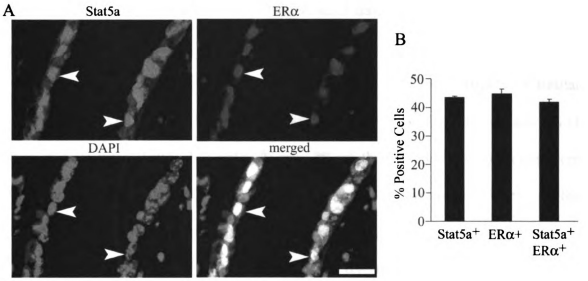


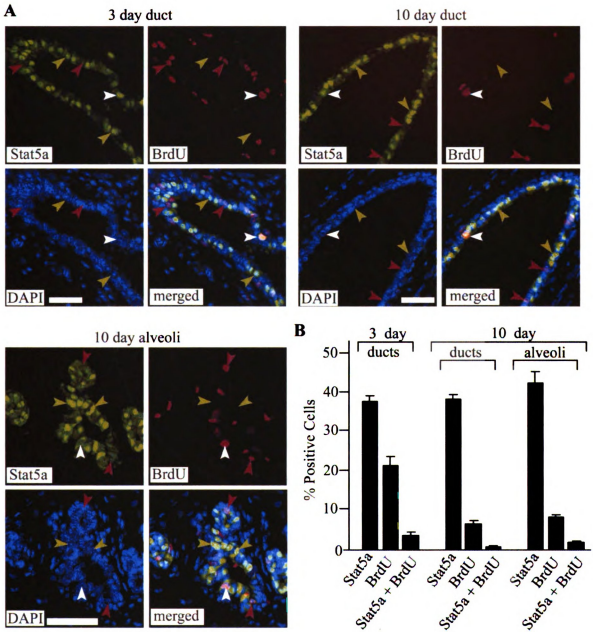
Figure 2.5. Colocalization of Stat5a with ERα. A. Staining for Stat5a and ERα was carried out on sections from 3 day E+P treated ovariectomized mice. White arrowheads indicate Stat5a and ERα positive cells. Scale bar = 20 μm. B. Quantitation of Stat5a and ERα colocalization in 3 day E+P treated animals. The values represent the mean ± SEM from three animals per group, with a minimum of 800 cells/mouse analyzed. Images in this dissertation are presented in color.

these hormones (14, 15). Since Stat5a is the predominant isoform in the mammary gland and Stat5a-/- mice display a more pronounced mammary gland developmental defect than Stat5b-/- mice, the effect on proliferation is likely due to the lack of Stat5a. To determine if $Stat5a^+$ cells proliferate in response to E+P, 3 and 10 day E+P treated animals were injected with BrdU 2 hours before they were euthanized, and tissue sections were double-labeled for BrdU and Stat5a. After 3 days of treatment, 38% of ductal epithelial cells were $Stat5a^+$, 21% were $BrdU^+$, and 3% were both Stat5a and BrdU positive (Fig. 2.6). Thus, only 9% of the cells expressing Stat5a were $BrdU^+$. A similar result was observed in the 10 day E+P treated mice, where 6% of ductal cells and 8% of alveolar cells were $BrdU^+$, but only 2% and 4% of the Stat5a expressing cells were $BrdU^+$, respectively (Fig. 2.6). When BrdU incorporation was compared between the three populations of cells (total, $Stat5a^+$ and $Stat5a^-$), $Stat5a^-$ cells were 3 to 6 times more likely to incorporate BrdU than $Stat5a^+$ cells ($p < 0.05$) after either 3 or 10 day E+P treatments. Thus, although some $Stat5a^+$ cells incorporate BrdU in response to hormone treatment, they are underrepresented in the pool of proliferating cells.

Colocalization of Stat5a and WAP or RANKL

The observation that Stat5a is expressed at significant levels in the virgin gland prompted the investigation of potential target genes at this developmental stage. Stat5a is a well-established differentiation factor that induces the expression of milk protein genes including WAP in the lactating mammary gland. Immunohistochemistry was used to investigate whether this gene was also induced in Stat5a expressing cells in E+P treated

Figure 2.6. Immunodetection of Stat5a and BrdU in mammary glands of hormone treated mice. Ovariectomized 19 wk old mice were treated with E+P for 3 or 10 days, and animals were injected with BrdU 2 hours before being euthanized as described in Materials and Methods. A. Representative structures from mammary glands showing Stat5a (green), BrdU (red), DAPI stained nuclei (blue), and merged images. Green arrowheads indicate Stat5a positive, BrdU negative cells. Red arrowheads indicate BrdU positive, Stat5a negative cells. White arrowheads indicate BrdU positive, Stat5a positive cells. Scale bar = 25 μm. B. Quantitation of Stat5a and BrdU colocalization in 3 and 10 day E+P treated animals. The values represent the mean ± SEM from three animals per group, with a minimum of 800 cells/mouse analyzed. Images in this dissertation are presented in color.


A
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BrdU
DAPI
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B
3 day
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ducts
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alveoli
% Positive Cells
50
40
30
20
10
0
Stat5a
BrdU
Stat5a + BrdU

mice. In agreement with previous studies, WAP staining was present on the luminal surface of alveoli when lactating mammary glands were examined (Fig. 2.7A), and a large percentage of the alveolar cells were Stat5a positive. As expected, both WAP and Stat5a were absent from mammary glands in control ovariectomized mice. Despite the induction of nuclear Stat5a in mammary glands from 3 day E+P treated mice, no WAP staining was detected. Thus, the presence of Stat5a is not sufficient to induce the expression of the milk protein, WAP, in the virgin gland.

In addition to its role in differentiation, Stat5a may also be a proliferation factor in the mammary gland. Since $Stat5a^+$ cells are less likely to proliferate than their $Stat5a^-$ neighbors (Fig. 2.6), it is likely that any proliferative activity of Stat5a is accomplished via a paracrine mechanism. One candidate paracrine factor is the TNF family member, receptor activator of NF-κB ligand (RANKL). RANKL is a secreted protein that is essential for normal mammary gland development (29). PRL induces RANKL expression in primary mammary epithelial cells, and experiments in heterologous cell culture models suggest that this is through the Jak2/Stat5a pathway (30). To investigate whether RANKL might be a Stat5a target *in vivo*, mammary gland sections from 3 day control or E+P treated mice were stained for both Stat5a and RANKL. Mammary cells from control mice lacked both Stat5a and RANKL staining (Fig. 2.7B), and both were induced after 3 days of E+P treatment. In addition, there was a high level of colocalization of the two proteins (Fig. 2.7B), supporting the hypothesis that they are functionally linked.

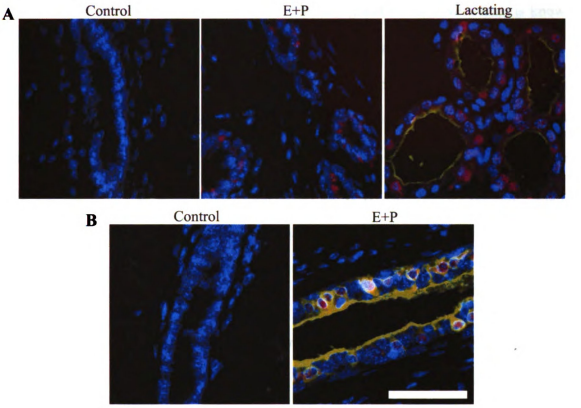

Figure 2.7. Immunodetection of Stat5a and WAP or Stat5a and RANKL. Tissue sections from 3 day E+P and control treated ovariectomized mice were stained for Stat5a and either WAP or RANKL. 10 day lactating mice were included as a control for WAP. A. Representative structures showing merged images of Stat5a (red), WAP (green), and DAPI stained nuclei (blue). B. Representative structures of merged images of Stat5a (red), RANKL (green), and DAPI stained nuclei (blue). Scale bar = 25 μm. Images in this dissertation are presented in color.

DISCUSSION

Despite the importance of Stat5a in mammary gland biology, very little is known about the mechanisms that regulate its expression in this tissue. To address this issue, we studied the pattern of Stat5a expression in mammary epithelial cells during normal development and in response to treatment with the ovarian hormones E and P. Immunofluorescent staining revealed that Stat5a was expressed at extremely low levels in the pre-pubertal gland. Expression increased at puberty, with 37% of luminal cells in ducts exhibiting bright nuclear staining. The percentage of Stat5a positive cells increased significantly in adult virgin ducts, and increased further in alveolar cells during pregnancy. It was at its highest level during lactation, where more than 80% of luminal epithelial cells had both nuclear and cytoplasmic staining, decreased dramatically during involution, and was then restored to near virgin levels. The increase in Stat5a expression at puberty suggested that ovarian steroid hormones induce its expression. This was confirmed by the finding that Stat5a levels dropped dramatically following ovariectomy, and treatment with E+P was necessary to restore it. While pregnancy levels of hormones were used in these studies, it is likely that lower concentrations are sufficient to support Stat5a expression since it was present at high levels in the adult virgin gland. Activation and expression of Stat5 are not affected by variations in circulating hormone levels during the estrous cycle in mice (18), suggesting that relatively low levels of E and P are required for the amount of Stat5a observed in the virgin gland.

To investigate the mechanism by which E and P induce Stat5a, we examined the pattern of ERα, PR and Stat5a expression by immunohistochemistry. After 3 or 10 days

of E+P treatment, virtually all Stat5a$^+$ cells also contained either PRA or PRB, which is consistent with a model in which PR directly or indirectly activates the Stat5a gene. Since the two PR isoforms are rarely coexpressed in the same cell in the mouse mammary gland (27), these findings further suggest that either isoform can induce Stat5a expression in this system. This differs from the situation in human T47D breast cancer cells, where progestin induced Stat5a mRNA in cells expressing PRB, but not those expressing PRA (22). One potential explanation for this difference is that we have examined Stat5a protein instead of mRNA. Our experiments do not therefore establish if the observed increase in Stat5a levels after E+P treatment is due to increased gene transcription, mRNA stabilization, mRNA translation, protein stabilization, or a combination of the above.

The fact that combined E+P treatment was required to induce Stat5a in ovariectomized animals indicated an important role for E in regulating its expression. Since PR levels decrease significantly following ovariectomy (28, 31), one role for E could be to induce PR, which would then activate the Stat5a gene. ERα is present in the almost all Stat5a$^+$/PRA$^+$ cells after 3 days of E+P treatment (Fig. 2.4), which is consistent with this hypothesis. An alternative possibility is that E and P, acting through their respective receptors, are independently required to induce Stat5a in PRA$^+$ cells. The situation in PRB$^+$ cells is more complex, since the majority of PRB$^+$ cells in the mammary gland do not contain ERα (28). However, E is required for high levels of PRB expression in 10 day E+P treated animals (28), and may therefore contribute to Stat5a expression in these cells by indirectly regulating PRB levels.

We also considered a model in which E acts indirectly by increasing systemic PRL. It is well established that PRL activates Stat5 via the PRLR/Jak pathway (8, 32, 33), and E induces PRL secretion from the pituitary gland (25, 26). To investigate if this pathway also induces Stat5a expression, we examined glands from PRL and PRL+P treated animals. Neither treatment reproduced the increase in Stat5a expression seen with E+P. However, when PRL secretion was suppressed by bromocriptine in E+P treated mice, Stat5a was still induced, but was localized predominantly in the cytoplasm. This leads us to propose a model in which E+P, acting through their receptors, induce Stat5a expression in luminal epithelial cells in the virgin mammary gland. The protein is then activated via the PRL/PRLR/Jak pathway, leading to its phosphorylation and nuclear accumulation. This model is consistent with the results of Nevalainen et al., who demonstrated that treatment with PRL 24 hours after hypophysectomy increased the level of phosphorylated Stat5 in mammary epithelial cells (18), but did not affect the level of total cellular Stat5. It is likely that ovarian function was maintained for 24 h after hypophysectomy, and that Stat5a was present but inactive. It is interesting to note that alternative mechanisms are likely to be responsible for the high levels of Stat5a expression during lactation, since E, P and PR all decrease dramatically at parturition (34-36).

The importance of Stat5a during pregnancy and lactation is well established (10, 11, 14, 15), and the presence of activated Stat5 in the adult virgin gland suggested that it also has a function prior to pregnancy (18). The current observation that Stat5a appears in epithelial cells at puberty, when the mammary gland is undergoing ductal development, suggests that it regulates growth or differentiation in the virgin gland. The absence of

Stat5a in terminal end bud cells argues against a role in ductal elongation, and suggests a possible role in differentiation or branching. The phenotype of Stat5a deficient mice should help to provide insight into its function. Although no overt mammary phenotype was initially noted in Stat5a-/- virgin mice (10, 11), one report using mammary transplants indicated that Stat5a-/- epithelium had maintained terminal end buds and reduced secondary branching at 8 weeks compared to wild-type epithelium (37). The reduction in branching suggests that Stat5a is required for the differentiation of cells that are programmed to become alveolar cells during pregnancy. Such a lack of alveolar progenitor cells could then account for the stunted alveolar development seen in pregnant Stat5a-/- mice. The alveolar-like structures that do exist in Stat5-/- mice do not express the normal alveolar differentiation marker Npt2b, and retain a marker normally seen only in ducts, NKCC1 (14, 15), indicating that even those alveolar structures that do arise are not fully differentiated. In addition to its potential role in differentiation in the virgin gland, Stat5 has been reported to positively regulate proliferation in the pregnant or E+P treated mammary gland (14, 15). The present results demonstrate that the majority of cells proliferating in response to E+P do not express Stat5a, indicating that this isoform could only stimulate proliferation through a paracrine mechanism.

In order to fully understand the role of Stat5a in the virgin mammary gland, it will be important to identify its downstream targets. The WAP gene is induced by Stat5a during lactation, but the protein not expressed at detectable levels in Stat5a$^+$ cells in the virgin gland. Thus, it is likely that novel Stat5a targets remain to be identified. One potential candidate is the secreted cytokine RANKL. RANKL expression in mouse mammary cells *in vivo* requires E, P, and PRL (Fig. 2.7 and (30)), and *in vitro*

experiments indicate that PRL is acting through Stat5a (30). Furthermore, Stat5a-/- and RANKL-/- mice exhibit similar mammary gland phenotypes (10, 11, 29), suggesting that the two genes may act in the same pathway. RANKL promotes cell cycle progression by binding to its receptor, RANK, and initiating a signaling cascade leading to the activation of the cyclin D1 gene (38). Our results demonstrate that RANKL colocalizes with Stat5a in the mammary epithelium (Fig. 2.7), which is consistent with the hypothesis that Stat5a induces RANKL, which then stimulates proliferation in adjacent, Stat5a$^-$ cells. However, it is also possible that Stat5a and RANKL are independently induced by E+P. A second candidate Stat5a target in the virgin gland is Wnt-4. Wnt-4 is required for ductal side branching, and has been implicated as a mediator of the paracrine effects of P in mammary gland development (39). Since Stat5a co-localizes with PR and is induced by P, it could be an intermediate in a PR/Stat5a/Wnt-4 pathway.

In summary, the experiments reported here identify a novel role for E and P as critical regulators of Stat5a expression in the pubertal and adult virgin mammary gland, and indicate that this regulation likely occurs at the cellular level through epithelial ERα and PR. The newly synthesized Stat5a is then phosphorylated via PRL signaling, leading to accumulation of the active transcription factor in the nucleus where it can regulate genes involved in differentiation and/or proliferation. Our results further indicate that any proliferative effects of Stat5a are through a paracrine mechanism, and suggest that RANKL may mediate these or other effects. A detailed analysis of the developmental phenotypes of Stat5a-/- mice, and identification of its target genes, will be required to fully elucidate its functions in the virgin mammary gland.

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CHAPTER 3

EXAMINING THE FUNCTION OF STAT5A IN THE VIRGIN MAMMARY GLAND AND ITS ROLE IN E+P INDUCED PROLIFERATION

ABSTRACT

Signal transducer and activator of transcription (Stat)5a is a critical regulator of mammary gland development. The majority of Stat5a studies has focused on its roles in the pregnant and lactating mammary gland. However, the expression of active Stat5a in mammary epithelial cells was demonstrated as early as 5 wks of age, suggesting a function in early mammary gland development. To date, very little is known about Stat5a's role in the mammary gland of nulliparous mice. In the present study, early mammary gland development was examined using Stat5a knockout mice. Virgin Stat5a-/- mice exhibited defective primary branching and side-branching, providing evidence that Stat5a regulates these processes. In addition, Stat5a-/- mammary glands displayed a defective proliferative response to E+P treatment. The pathway by which Stat5a induces proliferation was subsequently examined. It was previously demonstrated that Stat5a positive cells rarely proliferate, suggesting that it induces proliferation through a paracrine mechanism. One potential mediator, receptor activator of NF-κB ligand (RANKL), was examined. RANKL is a secreted factor that can induce mammary epithelial cell proliferation. Stat5a-/- mammary glands were defective in upregulating the expression of RANKL in response to E+P treatment. In addition, the nuclear localization of a critical RANKL target, Id2, was diminished in Stat5a-/- mammary cells. Thus, the activation of the RANKL signaling pathway is one mechanism by which Stat5a can induce proliferation.

INTRODUCTION

The mouse mammary gland undergoes the majority of its development after birth. This postnatal program of mammogenesis occurs in two distinct stages. The first is initiated by the surge of ovarian hormones at puberty and results in a ductal tree extending to the edges of the mammary fat pad. The second major stage of mammary development is seen during pregnancy, when massive proliferation of the epithelial compartment results in additional side-branches and the formation of alveoli at the ends of those structures. A combination of several signaling pathways acting together is necessary to produce a fully functional mammary gland.

Signal transducer and activator of transcription (Stat)5a is one of the critical regulators of normal mammogenesis. The Stat proteins are a family of transcription factors which transduce extracellular cytokine and growth factor signals to the nucleus (1, 2). Two Stat5 isoforms exist (a and b, collectively referred to as Stat5), which share ~96% identity at the amino acid level (3). However, Stat5a has emerged as the chief isoform involved in mammary development, likely because it is expressed at much higher levels in the mammary gland than Stat5b (4). The vast majority of Stat5 studies have examined its functions during pregnancy. Inactivation of Stat5 in mouse lines by germline deletion has provided much of the information on its function.

Stat5a knockout mice (Stat5a-/-) exhibit reduced alveolar development at parturition and fail to lactate (4, 5). Transplanted Stat5 knockout (Stat5-/-) epithelium into wild-type stroma incorporates less BrdU in response to an acute E+P treatment (6). Thus, Stat5 regulated proliferation occurs through a mechanism autonomous to the

epithelial cells. When Stat5 was conditionally deleted specifically in the mammary gland during pregnancy, fewer phosphorylated histone 3 (a marker of cell proliferation) positive cells were present compared to control glands, again demonstrating a function in proliferation (7). Consistent with these results, increased proliferation and a predisposition to mammary tumors was seen in transgenic animals expressing a constitutively active form of Stat5 [comprised of the amino acid sequences from position 1 to 750 of the Stat5 molecule, the TAD (positions 677–847) of Stat6, and the kinase domain (positions 757–1129) of Jak2] in the mammary gland (8).

Although Stat5 has been implicated in mammary epithelial cell proliferation, very little is known about the underlying mechanism by which it acts. Data shown in chapter 2 demonstrate that Stat5a positive cells rarely proliferate in response to E+P compared to adjacent Stat5a negative cells, strongly suggesting that a paracrine molecule may mediate Stat5a's effect in neighboring cells. To gain additional information about the role of Stat5a outside of pregnancy and the pathways by which it controls epithelial cell proliferation, a more detailed examination of mammary gland morphology in pubertal and mature virgin, as well as in hormone treated Stat5a-/- mice is presented here. These studies establish a role for Stat5a in normal primary branching and side-branching in the nulliparous gland, and a critical role in the proliferative response to E+P. Finally, we show a loss of Stat5a leads to reduced RANKL levels following E+P treatment, and propose that this protein is an important mediator of Stat5a regulated proliferation.

MATERIALS AND METHODS

Animals:

Mammary glands were obtained from pubertal (6 wk old) and adult (18 wk old) Stat5a-/-, Stat5a+/+ and Stat5a+/- female mice in the C57Bl6/129-svj mixed background. For hormone treatments, 18 wk old virgin mice were ovariectomized (OVX) and 1 wk after OVX animals were injected every 24 h for five days with E+P (1 μg + 1 mg, respectively, per injection) administered subcutaneously into the dorsum of the neck. Animals were injected intraperitoneally with 70 μg/g body weight of 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St Louis, MO) two hours before they were euthanized. All animal experimentation was conducted in accordance with accepted standards of humane animal care and approved by the All University Committee on Animal Use and Care at Michigan State University.

Tail DNA was prepared as described (9). Genotyping of mice was performed by PCR. For PCR analysis, the wild-type Stat5a allele was detected by using primer F9 (5'-AAG GGA CAG GAA GAG AGA AGG-3'), and primer R1 (5'-CCC ATA CAA CAC TTG CAT CT-3') located at either side of the hygromycin resistance gene insertion site. This primer pair amplifies a fragment of 274 bp from wild-type and heterozygous mice but not from Stat5a knockout mice. DNA was also amplified by using primers R1 and TKp; the latter is located within the hygromycin resistance vector used to disrupt the Stat5a locus (5'-GCA AAA CCA CAC TGC TCG AC-3') to detect the Stat5a knockout allele. In this case, a 110-bp fragment was detected in mice heterozygous or homozygous

for the Stat5a knockout allele, while no signal was detected in wild-type mice. Genotyping was confirmed by immunohistochemical analysis for Stat5a as described in Materials and Methods from chapter 2.

Whole mount analysis:

To perform whole mount analyses, the left inguinal mammary gland (gland # 4) was used. The excised glands were fixed in 10% phosphate-buffered formalin overnight at 4 C for 24 h, hydrated, stained overnight at 4 C in Carmine alum, dehydrated, cleared in xylene, and stored in methyl salicylate until ready for analyses.

Immunohistochemistry:

Inguinal mammary glands were fixed in 10% phosphate-buffered formalin overnight at 4 C, dehydrated, cleared and embedded in paraffin. Tissue was sectioned to 5 μm, mounted on 3-aminopropyl triethoxysilane-coated coverslips, and allowed to dry for 24 h at room temperature. The tissue was then dewaxed and rehydrated through a descending gradient of ethanol. Sections were autoclaved (121 C at 15 psi) for 20 min in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. Specific blocking and antibody incubation protocols are detailed below. Each incubation step was followed by two 5 min washes in PBS.

For RANKL, Id2, and PRA labeling, sections were blocked for 30 min in 2% bovine serum albumin in PBS, pH 7.3 (2% PBSA). Samples were first incubated with

goat α-RANKL antibody (R&D Systems, Minneapolis, MI), rabbit α-Id2 antibody (Santa Cruz, Santa Cruz, CA), or rabbit polyclonal α-PR antibody, which detects only PRA (Dako Carpinteria, CA) in 2% PBSA overnight at 4 C, then incubated with rabbit α-goat (for RANKL staining) or goat α-rabbit antibody conjugated to Alexa 488 (green)(for Id2) or Alexa 546 (red)(for PRA) (Invitrogen, Carlsbad, CA) in PBS for 30 min. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, Saint Louis, MO) and pictures were taken on a Nikon Eclipse fluorescent microscope (Tokyo, Japan) with MetaMorph software (Molecular Devices Corp., Downington, PA). For Id2 staining, nuclei were counterstained using TOPRO-3 iodide (Invitrogen), and visualized using a Pascal laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

For BrdU labeling, sections were first blocked with goat α-mouse IgG Fab fragments (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100 in 1% PBSA for 1 h, then blocked with normal goat serum (NGS) (Vector Laboratories, Burlingame, CA) diluted 1:1 in PBS for 30 min. The tissue was incubated with mouse α-BrdU antibody (provided as a kit from GE Healthcare, Piscataway, NJ) for 1 h. This was followed by 30 min with a biotinylated goat α-mouse antibody (Dako, Carpinteria, CA) (1:400) and ABC reagent (Vector Laboratories). Immunoperoxidase localization of antibody staining was achieved using 3'-3'-diaminobenzidene. The sections were counterstained with hematoxylin and visualized using a Nikon Eclipse 50i microscope and a Qimaging color camera with ImageQuant software (GE Healthcare).

Branch Quantitation and Statistical Analysis:

Primary branch point quantitation was achieved by examining one area of the #4 mammary gland whole mounts from 18 wk old mice. Using PowerPoint software, a rectangle of identical size corresponding to ~15% of the mammary gland area was aligned on photographs of each gland with similar lymph node placement. Primary ducts within the rectangle were traced, and branch points counted. The pixel area of each gland quantitated, as determined by the perimeter of duct ends, was calculated using ImageJ software (NIH, Bethesda, MA), and the number of branch points per gland was normalized to this area. This process was carried out for each mammary gland by three individuals, and the final data represents the average of these findings.

Sections examined for BrdU or PRA by immunoperoxidase and immunofluorescence methods were quantitated for the number of BrdU or PRA-positive cells with the aid of a light microscope (immunoperoxidase) or from captured images (immunofluorescence). Five mice per treatment were analyzed. A minimum of 1200 total cells and two independent sections per mouse were analyzed. Results are expressed as mean ± SEM, and differences are considered significant at $P < 0.05$ using Student's t test.

Immunoblot Analysis:

Whole mammary glands were obtained from 5 day E+P treated mice. The glands were minced and homogenized in a buffer of 50 mM Tris HCl pH 7.2, 6 mM MgCl2, 1

mM EDTA, 10% Sucrose (w/v) (1 ml/0.2 g mammary tissue) containing complete mini protease inhibitor cocktail tablet (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails I and II (Sigma, St. Louis, MO) using a Polytron homogenizer. Homogenates were centrifuged at 14,000 x *g* for 30 min and supernatants were used for immunoblots. Mammary gland extract (20 μg) was mixed with 1/10 volume of 10x SDS sample buffer with reducing agent and boiled for 5 min at 100 C. Protein samples were resolved on 12% polyacrylamide gels under denaturing conditions, and transferred onto Polyvinylidene difluoride membranes (Perkin Elmer, Walthan, MA). Membranes were blocked in 5% milk in phosphate-buffered saline with 0.5% Tween 20 overnight at 4 C and incubated with goat monoclonal α-RANKL (R&D Systems)(dilution 1:1000) overnight at 4 C. The secondary antibody was horseradish peroxidase-labeled rabbit α-goat (dilution 1:2000) (Biorad Laboratories, Hercules, CA). After 1 h incubation with secondary antibody, membranes were washed, incubated with Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL), and exposed to x-ray film.

RESULTS

Mammary gland morphology in pubertal and adult nulliparous mice

Previous studies of Stat5 in the mammary gland have focused heavily on its role in pregnancy related mammary development. The few reports concerning the morphology of virgin Stat5-/- and Stat5a-/- glands have been contradictory. While one study described a lack of side-branching 8 wks after Stat5a-/- epithelium was transplanted

into wild-type stroma (10), others reported no defects in intact, adult Stat5a-/- mice (4), or in outgrowths from transplanted Stat5-/- epithelium (6). However, the results presented in chapter 2 demonstrated that Stat5a is present and activated in mouse mammary epithelial cells as early as 5 wks of age, suggesting that it plays a role in the development of the virgin gland. To investigate potential functions of Stat5a in the non-pregnant mammary gland, whole mounts from 6 wk and 18 wk old Stat5a-/-, Stat5a+/- and Stat5a+/+ mice were compared. We observed that the ductal outgrowth in the 6 wk old pubertal glands had progressed to the same distance beyond the lymph node in all of the mice, regardless of Stat5a genotype (Fig. 3.1A), indicating that Stat5a is not necessary for ductal elongation. Although there was some heterogeneity observed between animals of the same genotype, the Stat5a-/- glands appeared to contain fewer ducts and TEBs, and looked less organized overall. Mammary glands from mature 18 wk old Stat5a-/- mice also had varying degrees of ductal morphology, but overall appeared less developed and organized than age-matched wild-type glands, with a clear paucity of side-branching (Fig. 3.1B). This finding supports earlier reports that Stat5a deletion in transplanted virgin mammary gland epithelium resulted in less side-branching (7), and emphasizes the specific requirement for the Stat5a isoform in early development. The heterozygote glands displayed a morphology intermediate between the Stat5a-/- and Stat5a+/+ mammary glands. The presence of this dosage effect demonstrates that maximum expression of the protein is required for normal mammary gland development.

In addition to the lack of side-branches, the 18 wk old Stat5a-/- glands appeared to contain fewer primary ducts. To test if the difference was significant, primary branch points were quantitated from similar regions of 18 wk old Stat5a-/-, Stat5a +/- and Stat5a

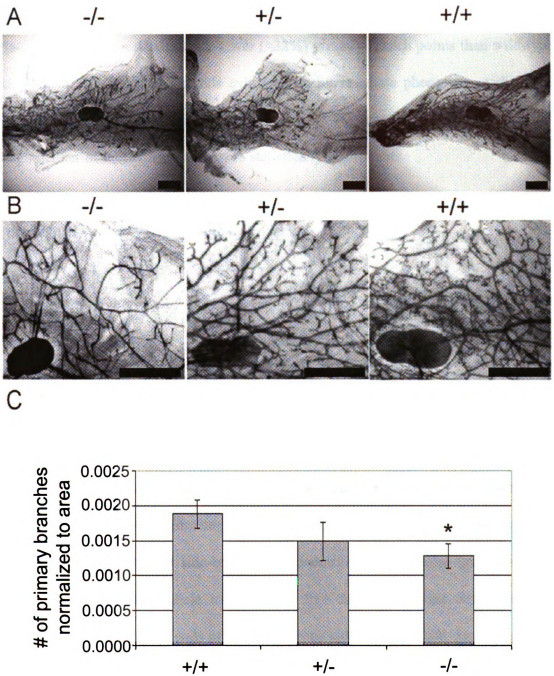


Figure 3.1. Whole mount analysis of pubertal and adult virgin mice. The fourth inguinal mammary glands were removed from Stat5a+/+, Stat+/- and Stat-/- mice and prepared as whole mounts. A. Mammary glands from 6 wk old pubertal mice and B. 18 wk old mature mice. Scale bar for all figures = 2 mm. C. Analysis of primary branch points in 18 wk old Stat5a+/+, Stat5a +/- and Stat5a -/- mammary glands. Quantitation was carried out as described in Materials and Methods for 3-9 animals per genotype, and is shown as the average of three individual analyses ± the standard deviation.

+/+ mammary glands as described in Materials and Methods. This analysis indicated that Stat5a-/- glands contain significantly fewer (~32%) primary branch points than wild-type glands (Fig. 3.1C). Stat5a+/- glands exhibited an intermediate phenotype, with ~21% fewer branch points than wild-type glands and ~14% more than knockout glands, although neither of these differences were statistically significant.

Morphological response to hormone treatment

To examine the role of Stat5a in mammary gland development, the response to E+P treatment was examined. 18 wk old virgin Stat5a-/-, Stat5a+/- and Stat5a+/+ mice were ovariectomized and allowed to recover for one week. Animals were then treated with a combination of E and P for five days. Previous studies have used two day treatments to examine the effects of Stat5 deletion on proliferation. However, a treatment period of two days is not sufficient to elicit a discernable morphological response. In contrast, five days of E+P treatment generates a visible response that includes enlarged tips at the ends of ducts and side-branches as a precursor to alveolar formation. As expected, mammary gland whole mounts from five day E+P treated wild-type mice displayed enlarged tips at the ends of their numerous side-branches and ducts (Fig. 3.2). Stat5a+/- and Stat5a-/- mice also exhibited enlarged tips at the ends of their side-branches and ducts. However, similar to the intact virgin mice, dramatically fewer branches were observed in the Stat5a-/- glands compared to the Stat5a+/+ glands. Again, the severity of this defect was variable between individual Stat5a-/- animals. The lack of branches from which alveolar budding can occur resulted in a corresponding decrease in the number of

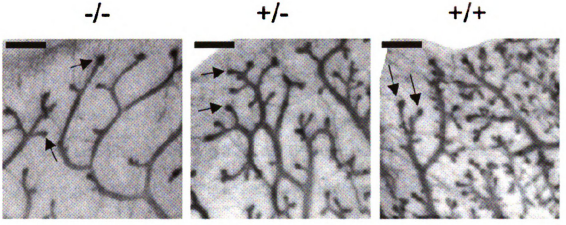


Figure 3.2. Morphological response of mammary glands to hormone treatment. Mammary gland whole mounts were prepared from 18 wk old OVX Stat5a+/+, Stat5a+/- and Stat5a-/- mice treated for 5d with E+P. Arrows indicate enlarged tips where alveoli will form. Scale bar = 1 mm.

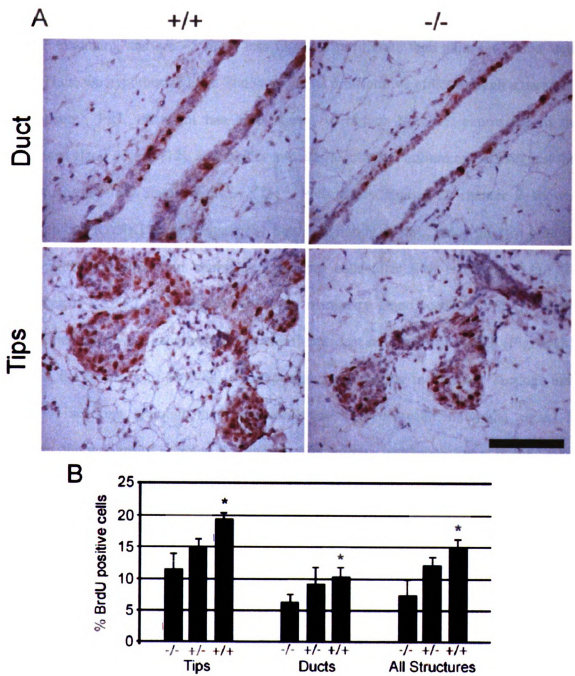
enlarged tips in the Stat5a-/- glands. Again, the Stat5a+/- mice displayed a phenotype intermediate between the knockout and wild-type animals. This finding suggests that the blunted response to E+P treatment is at least partially due to the pre-existing defect in side-branching observed in the virgin Stat5-/- mice.

Proliferative responses to hormone treatment

To determine if the proliferative response to E+P differed between the genotypes, mice were treated with a pulse of BrdU 2 h before they were euthanized. The analysis of BrdU incorporation in response to the 5 day E+P treatment is shown in Fig. 3.2. In the wild-type glands, 15% of all epithelial cells were BrdU positive ($^+$). Approximately half as many BrdU$^+$ cells (7%) were present in glands from Stat5a-/- mice. The percentage of BrdU$^+$ cells in heterozygous mice (12%) was higher than the knockouts and lower than the wild-types, but was not significantly different from either.

Although BrdU$^+$ cells were present throughout the entire mammary gland, the areas of highest proliferation were seen at the enlarged tips of ducts and side-branches, regardless of Stat5a genotype (Fig. 3.3). When duct and tip structures were analyzed independently, the difference in the percentage of BrdU$^+$ cells between the Stat5a genotypes was the same (Fig. 3.3B). Thus, fewer proliferating cells were present in all Stat5a-/- mammary gland structures compared to wild-type glands.

Figure 3.3. Proliferative response of mammary glands to hormone treatment. Immunoperoxidase detection of incorporated BrdU was performed on mammary gland sections from 18 wk old OVX mice treated for 5 d with E+P. A. Representative images from BrdU staining is shown for Stat5a+/+ and Stat5a-/- ducts and alveolar buds. Scale bar = 30 μm. B. Quantitation of the percent BrdU-positive luminal epithelial cells was carried out. Proliferation was significantly higher in the Stat5a+/+ glands compared to Stat5a-/- glands. Results are shown for quantitation carried out on duct and alveolar bud structures independently, as well as for all structures types combined. The values represent the mean ± SEM from five mice per group with a minimum of 1000 cells analyzed per mouse. Images in this dissertation are presented in color.


A
+/+
-/-
Duct
Tips
B
% BrdU positive cells
25
20
15
10
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0
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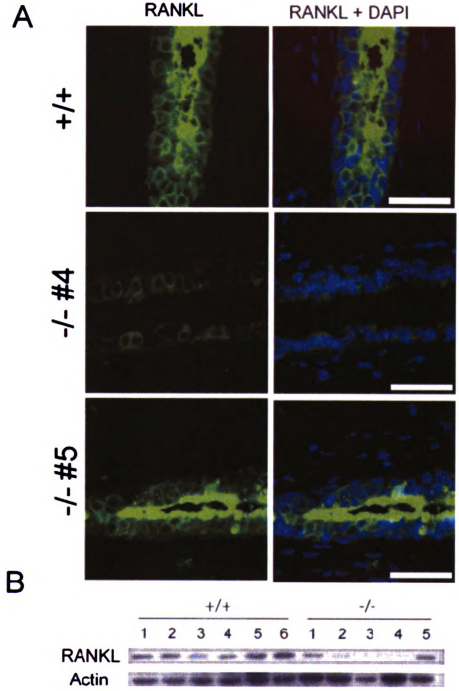
Tips
-/- +/- +/+
Ducts
-/- +/- +/+
All Structures

Downstream targets of Stat5a in the mammary gland

Data from chapter 2 demonstrated that Stat5a$^+$ cells rarely proliferate in response to E+P treatment, and are ~5 times less likely to proliferate than adjacent Stat5a$^-$ cells (11). Thus, we hypothesized that Stat5a elicits its proliferative effect through a paracrine mechanism. PRL treatment has been shown to induce RANKL expression in the mammary gland *in vivo* (12), and studies in heterologous cell culture models suggest this is through the Jak2/Stat5a pathway (13). Preliminary studies in chapter 2 showed evidence that RANKL is downstream of Stat5a signaling *in vivo*, since the two proteins were highly colocalized in mammary epithelial cells after E+P treatment (11). To determine if RANKL is regulated by Stat5a, mammary gland sections from the 5 day E+P treated mice were examined by immunofluorescent staining for RANKL (Fig. 3.4A). Overall, the intensity of RANKL staining was much weaker in Stat5a-/- sections than Stat5a+/+ sections, although there was some variability between individual mice. In addition, the amount of secreted RANKL in duct lumens was much lower in the Stat-/- mice. To confirm and quantitate these findings, Western blot analysis for RANKL in whole mammary gland homogenates from individual mice was carried out (Fig. 3.4B). In agreement with the immunohistochemical analysis, RANKL levels were variable between animals of the same genotype, particularly in the knockouts. However, 4 out of 5 Stat5a-/- mice had less expression of RANKL than Stat5a+/+ mice, confirming a defect. A strong correlation was observed between the immunofluorescent staining and immunoblot data from individual mice. For example among the five Stat5a-/- mice, the

Figure 3.4. RANKL expression in response to hormone treatment. Immunofluorescent staining for RANKL was performed using mammary gland samples from 18 wk old OVX mice treated for 5 d with E+P. A. Representative images of RANKL staining (green) is shown for Stat5a+/+ and Stat5a -/- ducts. Nuclei were stained with DAPI (blue). Images were captured with identical exposure times. Scale bar = 15 μm. B. Western analysis of RANKL levels in whole mammary gland homogenants from Stat5a+/+ and Stat5a-/- mice. Each lane represents a single mammary gland from independent mice (6 Stat5a+/+ mice and 5 Stat5a-/- mice). Images in this dissertation are presented in color.


A
RANKL
RANKL + DAPI
+/+
-/- #4
-/- #5
B
+/+
-/-
1 2 3 4 5 6 1 2 3 4 5
RANKL
Actin

lowest levels of RANKL were detected by both methods in mouse #4, while the highest levels of RANKL were detected by both methods in mouse #5.

The data shown here and in chapter 2 support the model that E+P induces expression of Stat5a, and activated Stat5a leads to RANKL expression. P treatment can induce the expression of RANKL in the mouse mammary gland (12), and knockout studies have shown that PR is necessary for the increased expression of RANKL in the mouse mammary gland after E+P treatment (14). To examine whether the defect in mammary cell proliferation or RANKL expression in Stat5a-/- mice was due to altered PR levels, staining for PRA was carried out on the 5 day E+P treated Stat5a-/- and Stat5a+/+ mammary gland sections. The two genotypes exhibited similar PRA staining intensities (Fig. 3.5A), and contained a similar percentage of PRA+ cells (~ 47%) (Fig. 3.5B). Thus, the altered response to E+P is not due to a lack of PRA expression, and these findings support the model that Stat5a can directly induce RANKL expression. Analysis of the PRB isoform was not carried out since this protein is not readily detectable in the mammary gland until ~10 days of E+P treatment (15).

We next examined a potential downstream target of the Stat5a → RANKL pathway, Id2. The Id proteins are inhibitors of basic helix-loop-helix (bHLH) transcription factors, since they contain a helix-loop-helix motif, but lack a DNA-binding domain (16). As a consequence, they act as dominant-negative regulators of bHLH transcription factors via heterodimerization with these proteins. Id2-/- mice exhibit defective alveolar development and lactation similar to RANKL-/- and Stat5a-/- mice (17, 18). Recently, it was reported that Id2 nuclear localization is induced by RANKL, and is critical for RANKL-mediated proliferation in primary mouse mammary epithelial

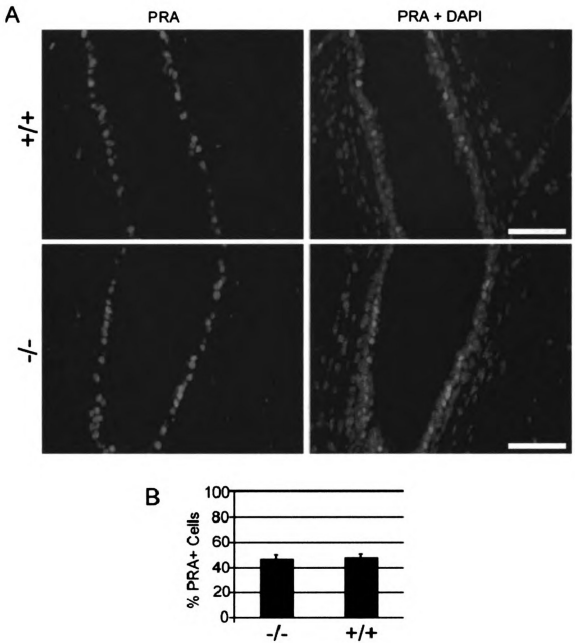


Figure 3.5. Progesterone receptor expression in response to hormone treatment. A. Immunofluorescent detection of PRA (red) in mammary gland samples from 18 wk old OVX mice treated for 5 d with E+P was performed. Nuclei were stained with DAPI (blue). Representative images from sections of Stat5a+/+ and Stat5a -/- mammary glands are shown. Scale bar = 30 μm. B) Quantitation of the percentage of PRA-positive epithelial cells was conducted. The values represent the mean ± SEM from five mice per group, with a minimum of 1000 cells analyzed per mouse. Images in this dissertation are presented in color.

cells (17). It is believed that Id2 mediates proliferation in mammary cells through its ability to inhibit transcription of p21, a cell cycle inhibitor (17). To examine the levels and cellular localization of Id2 in the mammary glands from 5 day E+P treated Stat5a-/- and Stat5a+/+ mice, immunohistochemical analysis was carried out. Id2 staining was predominantly nuclear in all wild-type animals (Fig. 3.6). In contrast, Id2 was largely cytoplasmic in sections from the Stat5a-/- glands that had low RANKL expression, especially mice 2-4 (see Fig. 3.5B) (staining from mouse 3 shown). Interestingly, Id2 localization appeared nuclear in mice 1 and 5 (staining from mouse 5 shown), which had levels of RANKL similar to wild-type animals. Thus, Id2 nuclear localization correlated with high RANKL levels in both Stat5a+/+ and Stat5a-/- mice.

DISCUSSION

Numerous studies have defined a functional role for Stat5a in the pregnant mammary gland. Data from chapter 2 demonstrated that Stat5a is expressed and active in the virgin gland, but very little is known about its function outside of pregnancy. Evidence from previous knockout studies was contradictory, with some reporting no phenotype in the virgin gland, and others reporting a defect in ductal branching. To resolve this issue and to examine potential functions of Stat5a in the virgin gland, mammary whole mounts from Stat5a-/- and Stat5a+/+ mice were compared at the pubertal (6 wk) and mature (18 wk) stages of development. The results demonstrated that Stat5a was dispensable for ductal elongation, but was necessary for normal primary branching and side-branching (Fig. 3.1). This finding presents an intriguing new role for

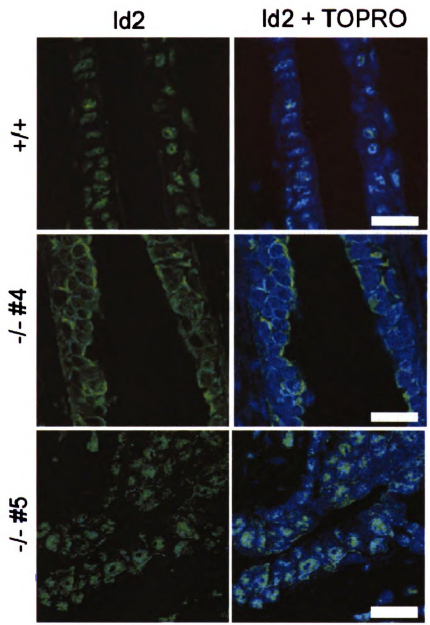


Figure 3.6. Id2 localization in response to hormone treatment. Representative confocal images are shown for immunofluorescent detection of Id2 (green) and nuclei stained with TOPRO (blue). The mammary glands shown correspond to Stat5a+/+ mouse #4 and Stat5a-/- mice #4 and #5 (see Fig. 3.4B) from 18 wk old OVX mice treated for 5 d with E+P. Optical slice = 0.5 μm. Scale bar = 5 μm. Images in this dissertation are presented in color.

Stat5a in mammary development. The mechanisms by which branching is regulated are not yet fully understood, but many interesting ductal morphogenesis phenotypes have been described that are associated with overexpression or deletion of specific genes. The regulators thus identified to date include hormones, growth factors and growth factor receptors, cell cycle regulators, transcription factors, cytokines and cell adhesion molecules (19).

It is interesting to note that while primary branching is accomplished mainly through the bifurcation of TEBs, Stat5a is not expressed in TEB structures (Fig. 2.1 of chapter 2) (11), suggesting a possible role for stromal Stat5a in this process. Stat5a signaling in leukocytes may explain its function in mammary branching. Macrophages and eosinophils have been shown to be located specifically to the stromal area surrounding TEBs in pubertal mammary glands, and are necessary for normal outgrowth and branching (20). These hematopoietic cells are recruited to TEBs by colony stimulating factor-1 (CSF-1) (20). Immunohistochemical and *in situ* hybridization studies have shown that mammary epithelial cells synthesize CSF-1 (17), while the CSF-1 receptor (CSF-1R) is exclusively expressed in macrophages in the mammary gland tissue of virgin mice (20). CSF-1 activation of the CSF-1R is capable of inducing Stat5a phosphorylation (21) and DNA binding (22). Furthermore, virgin CSF-1 knockout mice display a mammary phenotype similar to virgin Stat5a knockout mice, including limited primary branching and a disorganized ductal network (20). However, a role for epithelial Stat5a in primary branching should not be precluded since branching defects were reported in Stat5a-/- epithelial outgrowths in wild-type stroma (10). It is therefore

possible that multiple branching mechanisms are regulated by Stat5a in the mammary gland, and further investigation is needed to elucidate the specific pathways involved.

In contrast to primary branches, side-branches are produced via proliferation of epithelial cells in pre-established ducts. It was previously reported that Stat5 is necessary for normal mammary epithelial cell proliferation since the Stat5-/- knockout epithelium exhibited less BrdU incorporation after E+P treatment (6, 7). To investigate if this requirement was specific to Stat5a, 5 day E+P treated OVX Stat5a+/+, Stat5a+/- and Stat5a-/- mice were examined. The use of hormone treatments in OVX mice allowed us to avoid any possible complications due to altered endogenous ovarian hormone levels, and the 5 day treatments allowed us to examine the morphological response to hormone treatment. Although enlarged distal tips were present in glands from all genotypes, there was a notable lack of enlarged side-branch tips in the Stat5a-/- glands. This is likely due to the fact that there are fewer side-branches in Stat5a-/- glands to begin with, and therefore, fewer structures on which alveolar budding can take place. Thus, the defective alveolar development previously reported in pregnant Stat5a-/- mice might be at least partially the result of the side-branching defect seen in virgin mammary development. However, the defective E+P response in Stat5a-/- glands was not simply due to the scarcity of pre-existing side-branches. Structure specific analysis of proliferation demonstrated that the percentage of BrdU-labeled cells was lower in Stat5a-/- glands compared to Stat5a+/+ glands by ~40% in ducts and ~42% in tips, demonstrating that Stat5a regulates mammary epithelial cell proliferation throughout the entire gland. Although pregnancy levels of hormones were used in this experiment, it is

possible that the lack of side-branches seen in virgin Stat5a-/- mice could be due to a decreased proliferative response to E+P during the normal estrous cycle.

It was demonstrated in chapter 2 that Stat5a+ cells rarely proliferate in response to E+P treatment, suggesting that Stat5a induces proliferation via a paracrine mechanism. The data presented here indicate that Stat5a is required for the maximal induction of RANKL expression in response to E+P treatment. One important target of RANKL's receptor, RANK, in mammary epithelial cells is the inhibitor of bHLH transcription factors, Id2 (17). RANKL treatment induces the nuclear localization of Id2, which inhibits transcription of p21, and ultimately leads to cell proliferation (17). The nuclear localization of Id2 was decreased in the mammary epithelial cells in Stat5a-/- mice compared to Stat5a+/+ mice following E+P treatment. Along with data from chapter 2, this evidence supports a pathway where E+P → Stat5a → RANKL → RANK → Id2 → cell proliferation (see Figure 3.7). This model is consistent with the fact that RANKL-/-, Id2-/- and Stat5a-/- mammary glands display similar phenotypes during pregnancy, although the defective alveolar development seen in Stat5a-/- mice is less severe than the other knockout models (4, 5, 12, 23).

There was considerable heterogeneity observed in mammary gland morphology between individual animals of all Stat5a genotypes (+/+, +/-,-/-). This variability is likely to be at least partially due to the mixed background of the Stat5a mice. Likewise, the induction of RANKL was variable from gland to gland, especially in the Stat5a-/- mice. Along with the fact that the mammary defect is less severe in Stat5-/- mice than in RANKL-/- mice, this finding suggests that other pathways leading to RANKL expression may exist. Stat5b might be capable of inducing RANKL similar to Stat5a. It is also

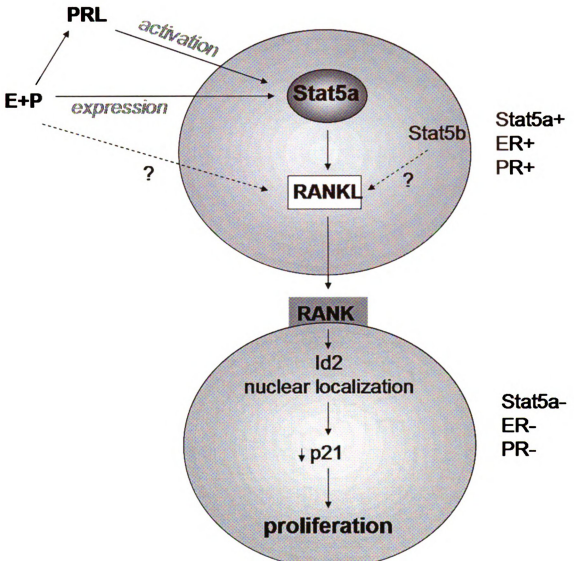


Figure 3.7. Proposed mechanism of Stat5a mediated proliferation in response to E+P. E+P treatment directly induces the expression of Stat5a, and indirectly activates Stat5a via PRL in ER+, PR+ mammary luminal epithelial cells. Activated Stat5a induces the expression of RANKL, which is secreted from the cell. RANKL acts through its receptor RANK on neighboring Stat5a-, ER-, PR- epithelial cells to induce the nuclear localization of Id2. Nuclear Id2 inhibits the expression of p21, leading to a proliferative response. In addition to Stat5a, other pathways may lead to the induction of RANKL in response to E+P treatment, such as Stat5b.

possible that PR might directly regulate RANKL, since PR-/- mice are defective in inducing RANKL following E+P treatment (14). The levels or activation of these and other molecules could potentially vary between animals due to their mixed background, causing a variable response to E+P treatment independent of Stat5a. However, this heterogeneity allowed us to draw some conclusions by examining individual mammary glands. Specifically, the strong correlation between RANKL expression levels and Id2 nuclear localization within individual animals supports the model of Stat5a-induced proliferation proposed here.

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CONCLUDING REMARKS

Stat5a is a signaling molecule shown to be critical for normal mammary gland development, and has been implicated in breast cancer. In this dissertation, I set out to explore two aspects of Stat5a biology in the mouse mammary gland. First, the regulation of Stat5a expression in mammary epithelial cells was examined. Although the pathways leading to Stat5a activation in the mammary gland had been elucidated, the mechanisms that regulate its expression in this tissue were relatively unknown. The results presented in chapter 2 demonstrated that the ovarian steroid hormones E and P are critical regulators of Stat5a expression in the pubertal and adult virgin mammary gland. Based on the fact that the majority of Stat5a+ cells also express ERα and PR, I argue that this regulation likely occurs at the cellular level through epithelial ERα and PR. E and P are sufficient to induce Stat5a expression, but activation and nuclear localization of the protein is dependent on PRL signaling, leading to the regulation of Stat5a responsive genes. This pathway offers an important new mechanism by which Stat5a signaling is regulated in mammary epithelial cells.

In chapter 2 of this dissertation, I demonstrated that Stat5a is present and activated in the pubertal and adult virgin mouse mammary gland. Although the function of Stat5a in the pregnant and lactating gland was well understood, its roles in the virgin gland were relatively unexplored. The data presented in chapter 3 revealed that Stat5a is required for both primary branching and side-branching in the virgin, developing mammary gland. Additionally, Stat5a-/- mice exhibit a defect in the proliferative response to E+P, and this may partially explain the lack of side-branches in the virgin gland. The results presented

in this dissertation also shed light on the mechanism by which Stat5a induces proliferation in response to E+P treatment. The finding that Stat5a+ cells do not themselves proliferate, suggest that they produce a paracrine mediator which can stimulate adjacent Stat5a- cells to proliferate. In chapter 3, evidence is presented that RANKL is one such paracrine mediator, and that it stimulates proliferation in responsive cells, at least in part, by inducing nuclear localization of Id2. In conclusion, the findings presented here represent a significant advance to the existing knowledge about Stat5a signaling, including insight into the regulation of its expression as well as the mechanism by which it induces proliferation during mammary gland development.

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APPENDIX

REGULATION OF STAT5A EXPRESSION BY R5020 IN PRIMARY MAMMARY EPITHELIAL CELLS

INTRODUCTION

Several lines of evidence suggest that progesterone (P) can regulate the expression of Stat5a in the mammary gland. Stat5a protein and RNA levels have been shown to increase during pregnancy, a period of increased systemic P levels. In addition, it has been demonstrated that P can directly induce the expression of Stat5a mRNA in the human breast cancer cell line T47D (1). Interestingly, the increased Stat5a expression in response to P treatment was much greater in cells expressing only PRB than cells expressing only PRA. While PRA is detectable in mammary epithelial cells (MEC)s in the virgin mammary gland, PRB is only detectable during pregnancy (2). Based on these facts, we hypothesized that P can induce expression of Stat5a in normal mouse mammary gland cells from mature adult mice to a greater degree than in cells from pubertal mice. This was tested by measuring the mRNA levels of Stat5a from MECs grown in a serum-free, three-dimensional *in vitro* culture system, treated with R5020, by quantitative RT-PCR.

MATERIALS AND METHODS

Reagents: Collagenase III and pronase were obtained from Worthington Biochemical Corp. (Freehold, NJ) and Calbiochem (La Jolla, CA), respectively. DMEM/Ham's nutrient mixture F-12 (1:1; DMEM/F12), Hanks' Balanced Salt Solution, and trypsin were purchased from Sigma (St. Louis, MO). L-Glutamine, penicillin, and streptomycin were purchased from Life Technologies, Inc. (Gaithersburg, MD). Rat collagen I (>90% pure) was purchased from BD Biosciences. The synthetic progestin,

R5020 (promegestone), was purchased from NEN Life Science Products (Boston, MA). Superscript III first strand cDNA synthesis kit and Trizol reagent was obtained from Invitrogen (Carlsbad, CA). DNA-free reagent was from Ambion (Austin, TX). Taqman probes and universal PCR master mix were obtained from Applied Biosystems (A/B) (Foster City, CA).

Mammary epithelial cells were isolated from pubertal (6 wk old) and adult virgin (18 wk-old) BALB/c female mice from our own colony using enzymatic dissociation methods as previously described (3). Cell viability was approximately 95% as determined by trypan blue exclusion. Freshly isolated epithelial cells (8×10^5/well) were suspended in neutralized collagen I (2 mg/ml, 600 µl/well), plated on top of a layer of collagen I (250 µl/well), and allowed to gel for 30 min at 37 C. Preparation of collagen gel was according to the manufacturer's instructions. All serum-free cultures were carried out in basal medium (BM): serum- and phenol red-free DMEM/F-12 supplemented with 0.1 mM nonessential amino acids (product 11140-050, Life Technologies, Inc., Grand Island, NY), 2 mM L-glutamine, 0.1 µg/ml insulin, 1 mg/ml fatty acid-free BSA (fraction V), 100 µg/ml penicillin, and 50 µg/ml streptomycin. Cultures were kept in 5% CO_2 at 37 C for 48 h. Cells were treated with 10 nM of the synthetic progestin, promegestone (R5020). R5020 was used instead of progesterone because it is metabolized less rapidly.

Primary cultures were flash frozen in liquid nitrogen and stored at -80 C. Frozen gels were homogenized with a Brinkman Polytron in Trizol reagent for ~3 min. RNA was extracted following the standard Trizol protocol. Quantitation of RNA was carried out using a Nanodrop spectrophotometer. Genomic DNA was eliminated by treating the

RNA with DNA-free reagent. Reverse transcription of the RNA was carried out using Superscript III enzyme, and the samples were treated with RNase H. Quantitative PCR was performed using commercially available Taqman assay probes and primer sets for Stat5a and 18s rRNA. Fluorescence was detected by an A/B 7900HT thermocycler, and data was analyzed on SDS software (A/B). 18S rRNA levels were used to normalize cDNA input amounts. Data represent the average of a minimum of 3 independent cell cultures, with cDNA from each culture analyzed in triplicate.

RESULTS

Results from these culture experiments showed a ~2-2.5 fold increase in the Stat5a mRNA level in response to a 48 h R5020 treatment. This finding was supported by a microarray analysis of primary cultures carried out independently of the experiments shown here. The microarray data demonstrated a similar ~2 fold increase in Stat5a expression when cells were treated with R5020 for 24 h (data not shown). Contrary to our hypothesis, cells from the pubertal and adult mice exhibited an equal increase in Stat5a mRNA expression in response to R5020. This finding is consistent with the *in vivo* data from chapter 2, which showed that Stat5a expression is induced by E+P treatment in cells containing either PRA or PRB.

The results from the culture experiments are not completely consistent with those from the *in vivo* studies. Expression of Stat5a *in vivo* required both E and P, while R5020 was able to induce Stat5a expression in the absence of E in the primary cell culture system. A possible explanation for this contradiction could be a decrease in the amount

of PR in the mammary glands following ovariectomy. To remove the confounding effects of systemic E and P, the mice were ovariectomized prior to hormone treatment. However, it has been demonstrated that ovariectomy results in decreased PR levels in the mammary epithelial cells (4). In contrast, the primary cells used in the culture studies were isolated from intact mice. P might be able to induce Stat5a expression *in vivo*, but could require a minimum amount of PR above the level present one wk after ovariectomy. PRA levels are increased in response to E treatment ovariectomized animals (4). Therefore, E treatment may have been required in the animal experiments from chapter 2 to increase the amount of PR above a threshold level.

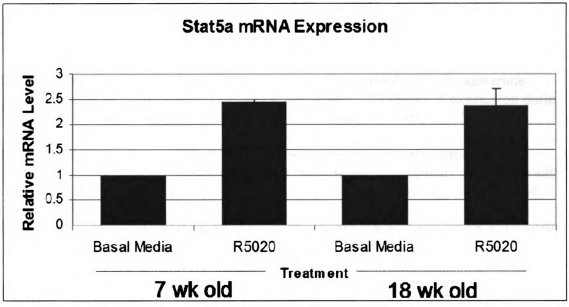

Figure 4.1. Stat5a mRNA expression in response to R5020. Mammary epithelial cells were isolated from pubertal (7 wk old) and mature (18 wk old) virgin mice and were grown as organoids suspended in collagen gel with Basal Media alone or with R5020 for 48 h. Total RNA was extracted from the organoids, RNA was DNaseI treated, and reversed transcribed. The cDNA was amplified using a Taqman probe and primer set for Stat5a. 18S rRNA was used to normalize cDNA input amounts. The data is expressed as the average fold induction over basal media from 3-5 independent cell culture experiments. PCR amplification was carried out in triplicate from a single cDNA preparation for each culture date.

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