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Developing a Mammary Gland is a Stāt Affair

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The mammary gland is a recent acquisition on the phylogenetic scale of organ evolution and is characterized by an unparalleled regenerative capacity. With each pregnancy an expanded lobulo-alveolar compartment rises on the ductal compartment and differentiates to secrete large amounts of milk during lactation. After weaning of the young the entire alveolar compartment undergoes apoptosis and is remodeled to return to a virgin-like state. Evolution recruited old hands from existing signaling pathways to guide and accomplish the extraordinary task of repeatedly building and destroying this highly specialized tissue. Seventy years ago it was known that the presence of estrogen, progesterone, and prolactin (PRL)[3] was essential for ductal and alveolar development. The recent ability to generate mice from which genes have been deleted by homologous recombination has made it possible to gain molecular insight into the signaling pathways used by these hormones to effect mammary differentiation. In the cast of characters progesterone and PRL are on center stage. After binding to its receptor, PRL activates the JAK-STAT pathway leading to transcription of genes which induce alveolar proliferation and differentiation. *In vivo* experiments have shown that JAK-Stat signaling is mandatory for adult mammary gland development and lactation. Two Stat molecules, Stat3 and Stat5, appear to have opposite functions and their relative activity may serve to control developmental cycles of mammary tissue. While Stat5 activity has been linked to alveolar proliferation and function, Stat3 activity correlates with the loss of alveolar function, cell death and the initiation of mammary tissue remodeling.

KEY WORDS: Prolactin signaling; Stat5; mammary development; alveoli; milk secretion.

INTRODUCTION

Historical Perspective

Classical research in the first three decades of this century firmly established that ovarian steroids and pituitary peptide hormones are mandatory for breast development and lactation. Alveolar development during pregnancy requires progesterone and, probably, a lactogenic hormone. At parturition the full process of milk protein synthesis and secretion is turned on. This process surely requires prolactin. In 1900 Halban made a good case for ovarian control of mammary growth (1). He demonstrated that oophorectomy was followed by mammary regression, and that transplantation of ovaries prevented the castration atrophy of the mammary glands. Twenty-eight years later Stricker and Grüter induced milk secretion artificially in castrated virgin rabbits by injecting a pituitary extract (2). With this simple, but consequential, experiment a new phase in mammary research began. In 1933, Riddle, Bates, and Dykshorn purified the hormone responsible for the milk secretion observed by Stricker and Grüter (3). They named it prolactin (PRL). In a landmark experiment, Topper and colleagues demonstrated in

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[3] **Abbrevations:** Prolactin (PRL); Janus kinase 2 (JAK2); signal transducer and activator of transcription (STAT); whey acidic protein (WAP); β-lactoglobulin (BLG); prolactin receptor (PRLR).

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1965 that synergistic signaling by insulin, hydrocortisone and PRL is required to produce casein in mammary organ cultures (4). The hunt for genes whose expression is controlled by PRL began with the advent of recombinant DNA technology and milk protein genes whose expression is controlled by PRL were isolated in the late 70s and early 80s (5–8). The development of transgenic animal technology was a milestone in our understanding that PRL induced gene expression is mediated by specific DNA motifs located within promoter sequences (9–14). Finally, the inactivation of genes within the mouse genome provided proof that three components of the PRL pathway, the PRL receptor and the transcription factors Stat5a and Stat5b, are mandatory for breast development and function (15,16). This line of experiments also demonstrated our ignorance of much of the complex biology encountered in the living organism. Although PRL acts in many tissues, and Stat5 is in the line of fire of many different signaling molecules, when they are absent *in vivo* an unprecedented level of specificity is revealed.

Prolactin Induced Transcription of Mammary-Specific Genes

Genes encoding milk proteins were first cloned in the early 1980's (5,6,8,17,18). In an important experiment Hobbs and his colleagues demonstrated in 1982 that the steady state levels of milk protein mRNAs increased several orders of magnitude during the course of pregnancy (6). By 1988, it was clear that promoter sequences from the whey acidic protein (WAP) and β-lactoglobulin (BLG) genes contain sufficient genetic information to target transcription exclusively to mammary tissue and to respond to PRL signals (19,20). In the quest for the identification of PRL response elements, a sequence (TTCNNNGAA) in the β-casein gene promoter was identified which conveyed PRL induced transcription in tissue culture cells (21). A phosphorylated nuclear protein from mammary tissue that bound to that sequence was named mammary gland factor (MGF) (21). MGF was cloned in 1994 by Wakao and Groner and based on its sequence, it was identified as a member of a specific family of signal transducer and activator of transcription (STAT) proteins and renamed Stat5. Although Stat5 RNA and protein have now been found in almost all tissues, the term MGF is valid since Stat5 activity in mammary tissue from lactating animals is at least an order of magnitude higher than in other tissues. Experiments in transgenic mice have now demonstrated that MFG/Stat5 binding sites in the WAP (9) and BLG (13,14) gene promoters are critical for maximal gene activity and PRL response *in vivo*. Several transcription factor binding sites have been identified in the BLG promoter (22). In addition to a nuclear factor 1 (NF1) and a NFκB site, three Stat5 binding sites were identified (13,14). The inactivation of a single Stat5 binding site had no effect on the induction of BLG expression in response to the lactogenic hormones prolactin, dexamethasone and insulin (13). However, the disruption of two Stat5 sites abrogated induction, and mutation of all three elements completely abolished hormonal responses.

The WAP gene is expressed almost exclusively in mammary tissue (20), and its transcription is induced several thousand-fold at mid-pregnancy and remains high throughout lactation (20,23). Induction and maintenance of WAP gene expression is mediated to a large extent through the prolactin and glucocorticoid signaling pathways (12,23). Transgenic studies have demonstrated that sequences conveying mammary specificity, and at least some of the hormonal responses, are located in the promoter and upstream region (20,24). The distal Stat5 binding site in the promoter is required for high level expression and PRL induction (9). The juxtaposed NF1 site is mandatory for WAP gene expression (9) and the promoter proximal Ets site mediates transcription at late pregnancy (25). Elements which convey the glucocorticoid response have also been mapped in the distal region of the WAP gene promoter (10).

Prolactin Activates the JAK-STAT Pathway

PRL is a 23 kDa peptide predominantly synthesized in lactotrophic cells of the anterior pituitary of vertebrates. A well-characterized role of PRL is its ability to induce lobulo-alveolar growth in the mammary gland and to stimulate post-partum lactogenesis (26). The synergistic approaches of biochemistry, physiology, molecular biology, and cell biology have unveiled molecular switches in PRL signaling pathways (Fig. 1). The cloning of the PRLR in 1988 (27) made possible the elucidation of the cascade of molecules linking the receptor to target genes (see (28) for an excellent review on the PRLR). PRL binding to its receptor leads to receptor dimerization and the activation of the Janus kinase 2 (JAK2), Fyn and the mitogen activated protein (MAP) kinase (29,30). Two catego-

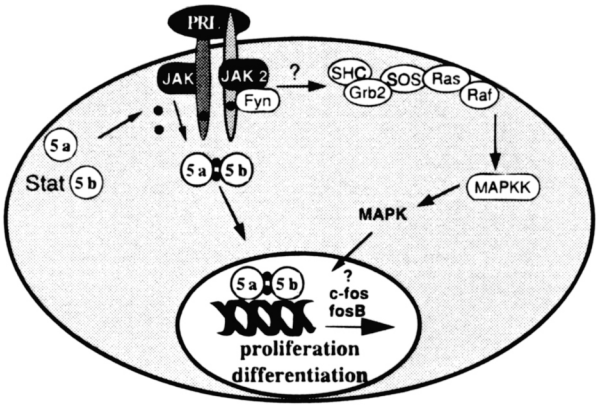

Fig. 1. Diagram outlining signaling pathways activated by PRL. In tissue culture cells PRL can signal through the MAPK and JAK-STAT pathways. PRLR, prolactin; STAT, signal transducer and activator of transcription; JAK, Janus kinase.

ries of PRL-induced biological effects can be distinguished, stimulation of cell proliferation and transcriptional activation of target genes determining the state of cell differentiation. Although PRL can activate the MAP kinase pathway in tissue culture cells, the significance of this pathway *in vivo* has not been confirmed. In contrast, *in vivo* experiments have demonstrated that signal transduction by the PRL receptor involves the JAK-STAT pathway (15). JAK2 phosphorylates Stat1, 3, and 5 on specific tyrosine residues (29,30). These activated Stat proteins bind to and induce transcription of genes containing γ-interferon activation sites (GAS) (TTCNNNGAA). However, gene deletions from the mouse genome have demonstrated that these three Stat members have different *in vivo* functions.

The ability of PRL to activate Stat5 was tested *in vivo*. The levels and activity of Stat5a and Stat5b in liver and mammary tissue were evaluated 15 minutes after PRL injection into mature virgins and day 8 pregnant females (Fig. 2). PRL injection did not overtly change the total amounts of Stat5a and Stat5b in either tissue (bottom row in Fig. 2). In contrast, both heterodimerization (indicated in the top row of Fig. 2) and phosphorylation of Stat5a and Stat5b sharply increased after PRL stimulation (Fig. 2). This demonstrates that PRL activates pre-existing Stat5a and 5b *in vivo*.

Dichotomy of Stat5 and Stat3 Activation: Is It a Matter of Life or Death?

The activation of overlapping but distinct sets of homo- and heterodimerizing Stat proteins is thought to contribute to their signal specificity (30). Circumstantial and experimental evidence suggest that Stat5 and Stat3 play different and perhaps opposing roles in the developmental cycle of alveolar proliferation, differentiation and death. While the activation of Stat5 is linked to alveolar proliferation and differentiation, Stat3 activity is most pronounced during alveolar cell death and mammary tissue remodeling. Although Stat5a and Stat5b proteins are present in similar amounts in mammary tissues of virgin, pregnant, lactating and involuting mice, the activated phosphorylated form is highly regulated (31). Levels of the

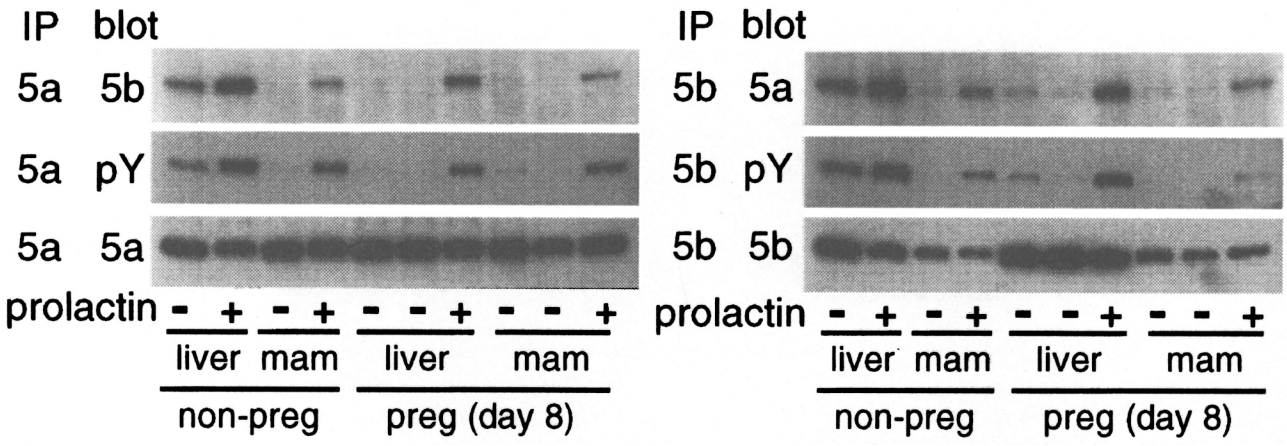

Fig. 2. Activation of Stat5a and Stat5b by PRL. PRL was injected intraperitoneally into mature virgins and day 8 pregnant mice, and mammary tissue was obtained 15 min. later from these mice (+) and from control mice injected with saline (−). Homogenates were incubated with either Stat5a or Stat5b specific antibodies as indicated (IP). The immunoprecipitates were separated by PAGE and analyzed for Stat5a/5b heterodimerization, Stat5a and Stat5b tyrosine phosphorylation (pY) and the presence of both Stat5 isoforms as described (15).

phosphorylated forms are very low in immature virgins, rise sharply during late pregnancy and decline rapidly during involution. Thus the induction of Stat5 phosphorylation during late pregnancy coincides with the functional differentiation of alveolar cells as indicated by the transcriptional activation of milk protein genes (Fig. 3). Experiments analyzing transient alveolar differentiation during estrus and in transgenic breast tumor models support the notion that the activated Stat5 is a differentiation factor (31).

While Stat5 signals life, Stat3 activation has been linked to mammary gland remodeling (31,32). Programmed cell death of mammary alveolar cells during involution commences within hours of the end of suckling. Locally, milk accumulates within alveolar lumina and, systemically, levels of lactogenic hormones fall. In order to differentiate the effect of declining hormone levels from local factors in the induction of programmed cell death Li and coworkers performed a series of teat sealing experiments (32). They demonstrated an opposite activity pattern of Stat5 and Stat3 during the shift of alveolar cells from differentiation to death, and thereby linked the JAK-STAT pathway to cell death. Three of the ten teats were sealed with a veterinary glue at day 10 of lactation preventing removal of milk although suckling was maintained. Despite continued systemic lactogenic hormone stimulation, stasis of milk within the gland induced alveolar programmed cell death within 24h. Thus, local signals are sufficient to induce programmed cell death. Most importantly, these results also showed that Stat3 and Stat5 activities are regulated independently of systemic hormones. Phosphorylation of Stat5a and Stat5b was lost with a concomitant loss of heterodimerization in the sealed glands, while both phosphorylation and heterodimerization were maintained in ipsilateral open

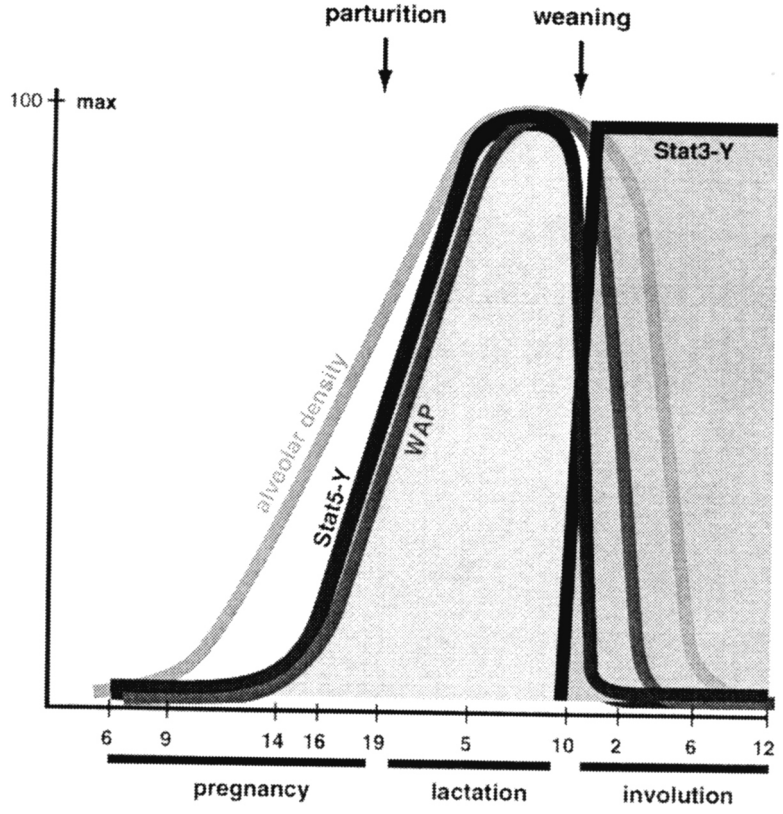

Fig. 3. Activation of Stat3 and Stat5 during mammary development. The phosphorylation of Stat3 and Stat5 in homogenates of mammary tissue was measured. The steady state levels of WAP RNA were determined by northern blots and liquid hybridization (6). Alveolar density was estimated by scanning mammary gland #4 in the mouse. The maximal activity of each parameter was arbitrarily set at 100. The numbers reflect the day of each particular stage of development.

glands. Strong phosphorylation of Stat3 appeared in the sealed gland by 12h again indicating that local factors alone can stimulate Stat3 activation during involution. Programmed cell death thus coincides with a loss of activity of the prolactin signaling molecules Stat5a and 5b and activation of Stat3. We therefore hypothesize that within the mammary gland Stat5 signals life whereas Stat3 is a death signal. The availability of Stat3-deficient mice should permit us to address this hypothesis.

Genetic Disruptions of the PRL Pathway

Our knowledge of the roles of Stat5a and Stat5b in PRL, GH, and cytokine signaling comes mainly from tissue culture cells. Such observations fall short of uncovering the roles of PRL and Stat5a and Stat5b in physiological processes *in vivo*. The establishment of mice in which the genes encoding the PRLR (16), Stat5a (15), and Stat5b (38; Eklund and Ihle, unpublished) have been inactivated, has now provided clear insight into their biology. Because the PRLR is expressed in multiple organs of the developing fetus and Stat5a and Stat5b are present in many signaling pathways, late fetal or neonatal lethality might have been expected in the absence of these proteins. In contrast to this widespread anticipation, mice deficient in the PRLR or in Stat5a or Stat5b were born and survived until adulthood. Defects were confined to a few tissues and specific physiological conditions.

Females with only one intact PRLR allele failed to lactate after their first pregnancy due to greatly reduced mammary development (16; see Ormandy *et al.* (33) for a review on this subject). Epithelial cell proliferation during pregnancy appears to depend on a threshold of PRLR which cannot be obtained with just one functional allele. However, mammary gland development after the second pregnancy or in older nulliparous females was sufficient for successful lactation, demonstrating that continued hormonal stimuli will eventually lead to development of a functional gland. In contrast, the progesterone and estrogen receptors, although essential for mammogenesis, require only one functional allele to mediate complete development (see 34; Bocchinfuso and Korach (35); Cunha *et al.* (36) for review).

The most noticeable phenotype of Stat5a-deficient mice (15) is their inability to lactate due to a failure of the gland to develop fully and to differentiate during pregnancy (Fig. 4). Two distinct and perhaps separable phenotypes were noted in the Stat5a-deficient mice. First, there was a reduction in the number of alveoli and, second the alveoli failed to undergo functional differentiation (for a diagram see Fig. 5). The superimposable expression pattern of Stat5a and Stat5b during pregnancy and lactation (15) in conjunction with the 96% sequence similarity between the two isoforms (15) suggested that Stat5b might be able to compensate for the loss of Stat5a. However, Stat5b protein levels, and even more pronounced, the extent of phosphorylation, were greatly reduced in Stat5a-deficient mammary tissue indicating that efficient phosphorylation of Stat5b requires the presence of Stat5a. The mechanism is not clear, but it is possible that activated Stat5a is required to achieve and maintain that state of cell differentiation required for full activation of Stat5b. There are two plausible ways to explain the lack of compensation by Stat5b. First, Stat5b may have an unique biochemical activity distinct from Stat5a. Alternatively, a certain threshold level of phosphorylated Stat5 (either a or b) may be

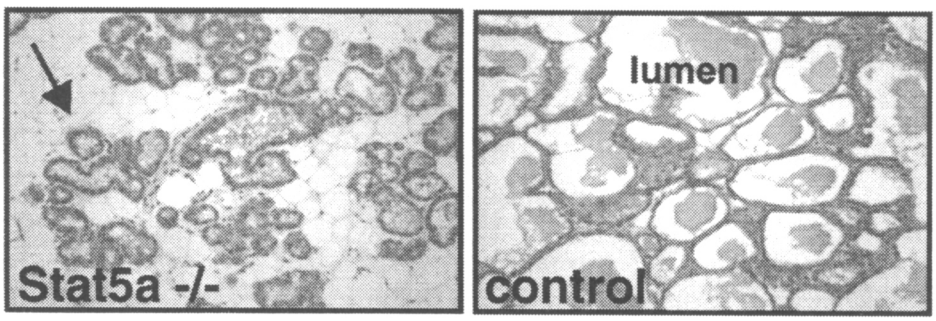

Fig. 4. Histological analysis of mammary tissue from partum Stat5a-deficient and control mice. The arrow points to an alveolus. The lumen of an alveolus from a control mouse is indicated. Note that the alveolar lumina in Stat5a-deficient mice fail to expand.

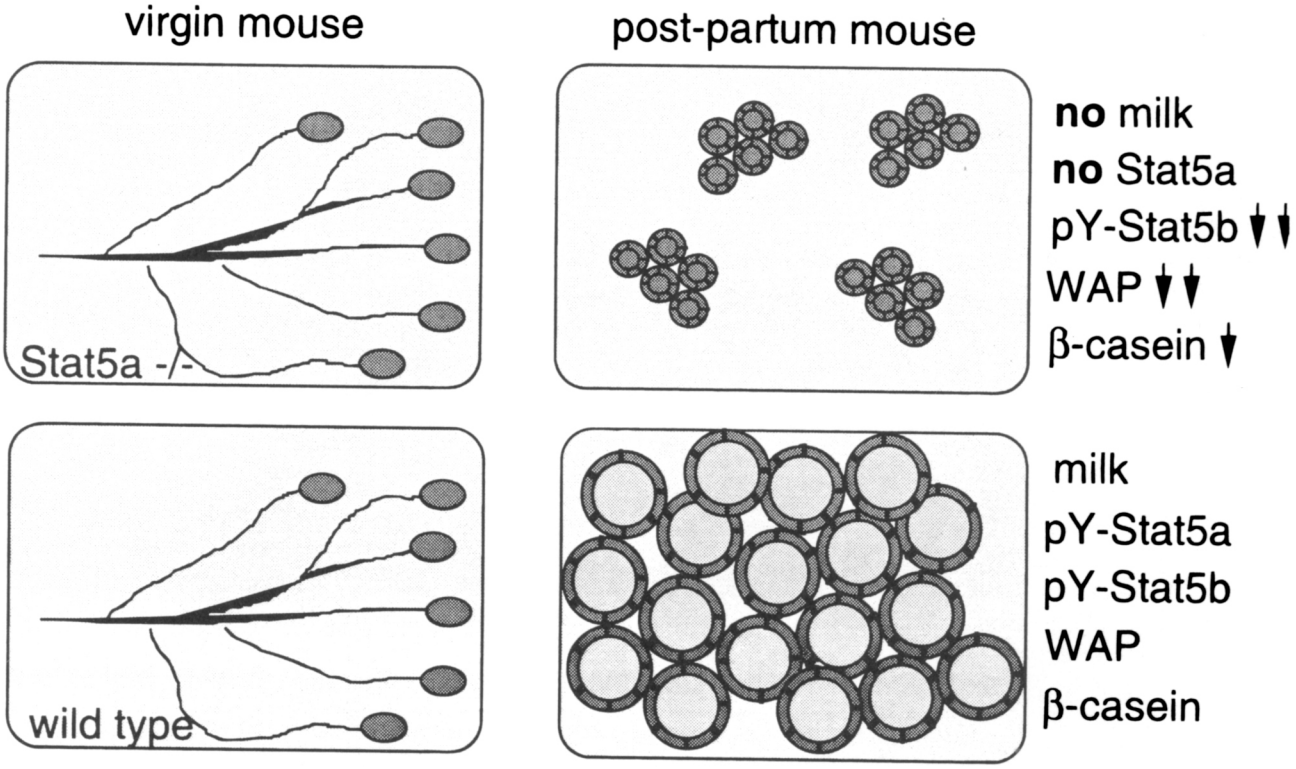

Fig. 5. Diagram of the developmental and functional defects observed in Stat5a-deficient mice. Ductal outgrowth during puberty appeared to be unaffected. Late pregnant and post-partum Stat5a-deficient mice exhibited a lower alveolar density than their wild-type litter mates. The alveoli failed to expand and milk was not detected in the lumina. Phosphorylation of Stat5b and expression of the WAP gene were sharply reduced in Stat5a-deficient mice. Steady-state levels of β-casein mRNA were slightly reduced.

required for functional mammary development. The delayed development seen in PRLR-deficient mice is consistent with this hypothesis. On the other hand, Stat5a and Stat5b have unique and nonoverlapping activities as suggested by their different C-termini, which encompass a transcriptional activator sequence (37).

Whether Stat5a and Stat5b have unique functions should also be addressed by the derivation of Stat5b deficient mice. Two groups have recently obtained such mice although the preliminary results appear to be different. In one case (38) the phenotype of the Stat5b-deficient mice is distinct from that of the Stat5a-deficient mice. These mice exhibited reduced growth in males and severely compromised fertility in females. Interestingly, the reproductive lesions in Stat5b-deficient mice were similar to those observed in PRLR-deficient mice. Observation of this phenotype suggested that prolactin signaling through Stat5b is a key regulator of mammalian reproduction. A second group (Eklund and Ihle, St. Jude) obtained a slightly different phenotype. The reproductive impairment was less severe, no size difference in the males was observed, and the females could lactate. The basis for the difference in phenotypes between the two Stat5b null mice is currently unclear. As in other knock-out mice it will be essential to characterize multiple, independently derived strains. Preliminary analysis of pre-partum and post-partum mammary whole mounts from the St. Jude mice indicates that alveolar density is greater than in the Stat5a-deficient mice (Eklund, Liu, Robinson, Hennighausen, and Ihle, unpublished). Interestingly, Stat5a phosphorylation appears to be normal in Stat5b-deficient mice.

Stat5/MGF was originally identified as a transcription factor that binds to a specific site in the β-casein gene promoter and mediates the activation of a β-casein reporter gene in tissue culture cells (21,39). However, in the Stat5a-deficient mice, which have greatly reduced levels of phosphorylated Stat5b, transcription of the β-casein gene is only slightly diminished (15). Based on these *in vivo* results it may be necessary to reconsider the role of Stat5 in the activation of the β-casein gene. In contrast, transcription of

the WAP gene, which also contains a specific STAT-binding site, was strongly reduced in Stat5a-deficient mice (15)

The past 60 years of PRL research have been characterized by a synergy between biochemistry, genetics and physiology which culminated in the identification of molecular switches in the PRL signaling pathway. Future research will focus on the action of these switches in controlling the physiology of the mammary gland. Progress in understanding the cell- and ligand-specific effects of signaling molecules with a widespread distribution, such as the PRLR and the transcription factors Stat5a and Stat5b, will depend on our ability to further manipulate the genetic components of the mouse (see the perspective by Furth (40).

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