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Stat5 assumes distinct functions in mammary gland development and mammary tumor formation

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1. ABSTRACT

The mammary epithelium comprises luminal and basal cells which originate from multipotent mammary stem cells (MaSCs). They form ductal structures embedded in the mammary fat pad in virgin mice and differentiate during pregnancy into alveoli under the control of hormones and growth factors and the activation of specific transcription factors. Genetic manipulations of embryonic stem cells and the derivation of transgenic mice allowed the study of regulatory genes in mammary epithelial cells of particular differentiation states. We describe an alternative approach to investigate stage dependent gene functions in transgenic mammary glands based on *ex vivo*, genetically manipulated MaSCs and the reconstitution of functional epithelium upon their transplantation into cleared fat pads. Modification of MaSCs with Stat5 suppressing shRNA or a constitutively active variant of Stat5 showed that Stat5 assumes essential roles in alveolar lineage commitment, proliferation, differentiation and survival. Its persistent activation during post-lactational involution causes the formation of non-metastatic adenocarcinomas, resembling the human luminal breast cancer subtype. The tumor cells express estrogen and progesterone receptor (ER^+PR^+) and activated Stat3 and Stat5. They could become valuable to assess the therapeutic potential of anti-estrogens, aromatase inhibitors and Stat3 and Stat5 inhibition on tumor growth.

2. THE REGULATION OF MAMMARY GLAND DEVELOPMENT

2.1. Mammary gland development and mammary stem cells

The mouse mammary gland is similar in its structure and function to the human breast and represents a valuable model for the study of organogenesis and tumorigenesis. It contains epithelial and stromal components and develops mainly postnatally during puberty, pregnancy, lactation and involution accompanying the weaning of the offspring. Systemic hormones, local growth factors and cellular interactions regulate these developmental phases (1-3). Classical hormone ablation and replacement studies demonstrated the essential role of ovarian and pituitary hormones in the mammary epithelium growth and differentiation (4, 5). The generation of transgenic mouse models has helped to resolve the functions of the downstream effectors of these hormonal signaling (6-10). The epithelial and stromal components respond to signals that control the specification and cell fate of mammary stem and progenitor cells (11-15). Luminal epithelial cells surrounded by basal myoepithelial cells cooperate to form ducts embedded within the stromal components of the mammary fat pad consisting of adipocytes, vascular endothelial cells, fibroblasts and immune cells. Ductal outgrowth is regulated through the

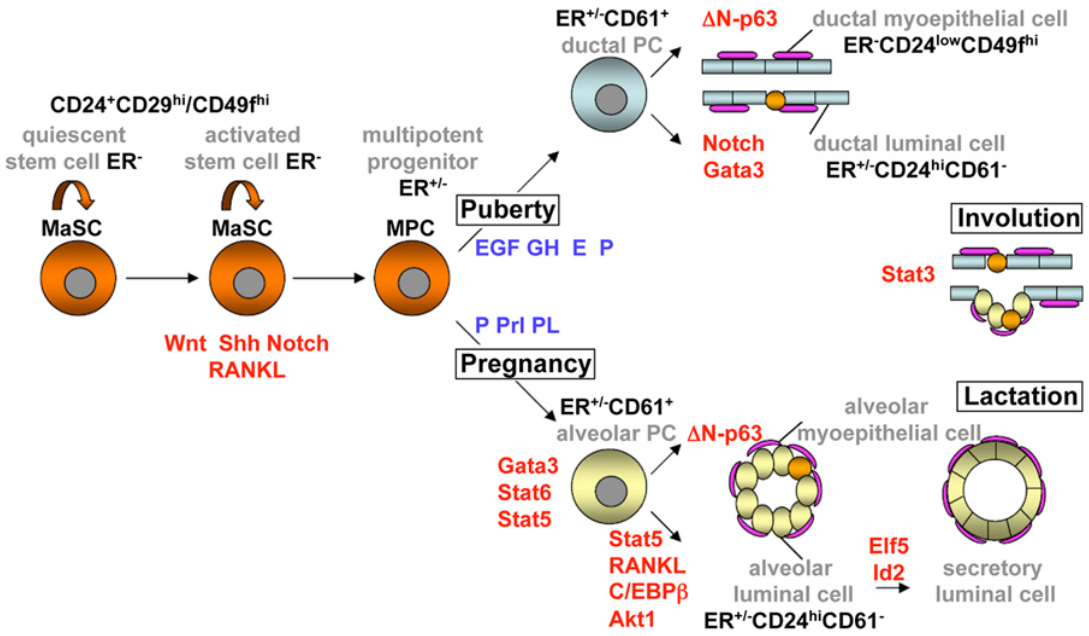


Figure 1. Regulation of lineage commitment in mammary epithelium by hormones, growth factors and transcription factors. Mammary stem cells (MaSCs) undergo asymmetric division and give rise to progenitor cells ($ER^{+/-}$). These multipotent progenitors (MPCs) develop into either ductal or alveolar cells in response to different hormones and growth factors (blue). Several signaling pathways and transcription factors (red) play key roles in the regulation of epithelial lineage commitment in the mammary gland. (CD24) heat stable antigen; (CD29) integrin-beta1; (CD49f) integrin-alpha6; (CD61) integrin-beta3; (C/EBP-beta) CCAAT/enhancer binding protein-beta; (deltaN-p63) terminus-truncated splicing variant of transformation related protein 63; (E) estrogen; (EGF) epithelial growth factor; (Elf5) E74-like factor 5; (ER) estrogen receptor; (Gata3) GATA binding protein 3; (GH) growth hormone; (Id2) inhibitor of DNA binding 2; (P) progesterone; (PL) placental lactogen; (Prl) prolactin; (PC) progenitor cell; (RANKL) receptor activator of nuclear factor kappa-B ligand; (Shh) sonic hedgehog signaling.

reciprocal interactions between the epithelial and the stromal cells (16-18).

During puberty, local factors like epidermal growth factor supplied by the mammary stroma and systemic hormones such as estrogen and growth hormone control early ductal outgrowth and elongation. This ductal tree undergoes progressive estrus-induced side-branching and maturation. Additional lateral buds emerge from the primary duct and form secondary and tertiary ductal side-branches. Progesterone (P) signaling plays an important role in ductal side-branching and alveologenesis (19). Paracrine factors such as Wnt4 operate downstream of progesterone receptor (PR) and mediate the process of side-branching (20).

In early pregnancy, P stimulates proliferation and alveolar morphogenesis. In addition, it inhibits terminal differentiation and represses milk protein gene expression (21). Ductal side-branches terminate in alveolar units, which are important for the establishment of alveolar structures during pregnancy. Alveolar differentiation comprises secretory initiation at mid-pregnancy and milk secretion at late-pregnancy. The lactation phase is accompanied by a decrease in circulating P, closure of tight junctions and the secretion of milk and lipid droplets into the alveolar lumen. Prolactin (Prl) and glucocorticoids stimulate transcription of the beta-casein milk protein gene through interaction of the transcription factor signal transducer and activator of transcription 5A (Stat5a) and the co-activator glucocorticoid receptor at the beta-casein promoter (22). Milk ejection and lactation last for about three weeks in mice.

Following weaning, the lobuloalveolar mammary compartment undergoes extensive remodeling. During involution the gland nearly returns to its pre-pregnancy state through apoptosis of secretory alveolar cells. Involution occurs in two distinct phases. During the first three days, a reversible phase is characterized by apoptosis of the secretory alveolar epithelium without major changes in glandular architecture. The second phase of involution is irreversible and associated with protease-mediated degradation of the basement membrane and re-differentiation of the adipocytes. Stat3 is an essential mediator of the first phase of involution and it regulates apoptosis by inducing expression of distinct PI3K regulatory subunits to downregulate PI3K/Akt-mediated survival signaling (23, 24). Recently, it has been shown that involution is accomplished through lysosomal-mediated cell death controlled by Stat3 (25).

The repeated expansion and regression of the mammary epithelium during subsequent rounds of pregnancy and lactation are based on the presence of adult mammary stem cells in this tissue. These stem cells are

able to self-renew and to give rise to progenitors cells of different lineages, which differentiate into functional progeny (26). The functional indication for these stem cells is based on transplantation experiments in which ductal fragments were used to reconstitute the glandular tree (27, 28). A small number of epithelial cells isolated from a mouse mammary gland at any developmental stage can regenerate a functional gland when transplanted into the cleared fat pad of a recipient mouse. This allows the study of stem cell functions under the influence of a normal physiological and hormonal environment. Particular cellular surface markers, CD24 (heat stable antigen), CD29 (beta1-integrin) and CD49f (alpha6-integrin) were used to enrich the stem cell population (29, 30). The laminin-binding integrin subunits beta1- and alpha6-integrins are highly expressed in cells located on the basement membrane such as myoepithelial and putative stem cells. Cleared fat pad transplantation experiments with the Lin^- $CD24^{med}CD49f^{high}/CD29^{high}$ cells showed that this subpopulation is enriched in mammary repopulating units.

The stem cell microenvironment provides a niche which maintains their stemness and prevents them from differentiating. This niche changes depending on the developmental stage and the hormonal milieu (31). In both virgin and pregnant glands, MaSCs reside in a specialized microenvironment and systemic reproductive hormones mainly determine their activation (32, 33). Stem cells were most frequently found in the $CD24^{low}ER^-$ basal cell fraction (34). It has been proposed that basal, ER^- stem cells divide asymmetrically and give rise to luminal ER^+ progenitor cells (35) which secrete paracrine factors in response to estrogen stimulation and induce the proliferation of ER^- stem cells.

2.2. Transcription factor regulation of mammary lineage commitment

Mammary gland development and alveologenesis is dependent upon the specification and maintenance of cell fates in multipotent MaSCs and their progenitors. This occurs through the activation of gene regulatory networks, which are composed of hierarchical sets of transcriptional activators and repressors (Figure 1). The mechanisms that regulate the derivation of particular progenitor cells from stem cells and their lineage commitment during mammary development involve the functions of specific transcription factors and transcription factor associated factors like the promyelocytic leukemia protein (PML).

The Notch signaling pathway has been implicated in luminal lineage commitment (36) whereas terminus-truncated splicing variant of transformation related protein 63 (detaN-p63), is involved in the commitment of the basal mammary epithelial cell lineage (14, 15). The transcription factor Gata3, a downstream effector of Stat6, has been shown to be essential for terminal end bud formation and ductal outgrowth and resembles the ER-alpha in its function. Gata-3 binds to the promoter of the forkhead transcription factor FOXA1, which serves as a pioneer factor for ER chromatin binding sites (37). Gata-3 thus controls important cell fate decisions in response to estrogen during ductal morphogenesis. It promotes the differentiation of lineage-restricted progenitor cells and is required for the commitment of the luminal lineage and the maintenance of luminal cell differentiation (11, 12).

PML is a tumor suppressor protein which is regulated by Stat transcription factors. It is found as a translocation product in the majority of acute promyelocytic leukemias (APLs). The PML-RARa oncofusion protein acts as a transcriptional repressor insensitive to ligand induction and interferes with gene expression programs involved in differentiation, apoptosis and self-renewal (38). In mammary cells PML determines the balance between luminal progenitor populations, the cell lineage determination of bi-potent luminal progenitor cells and is required for functional differentiation of alveolar cells (39).

Proper alveolar differentiation also requires the transcription factors such as Stat5a, CCAAT/enhancer binding protein (C/EBP-beta), Akt1, basic helix-loop-helix protein inhibitor of DNA binding 2 (Id2), nuclear factor kappa-B (NF-kappaB) and E74-like factor 5 (Elf5). These genes can regulate the alveolar cells by controlling alveolar cell fate decision, and alveolar progenitor cell proliferation, differentiation and survival as well as the maintenance of alveolar cell differentiation.

C/EBP-beta belongs to the bZIP transcription factors and is important for the maintenance of the MaSCs repopulating ability and it controls luminal cell lineage commitment and induces the proliferation of alveolar progenitor cells (40, 41).

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling in mammary gland play a central role in the regulation of cell proliferation, motility, glucose homeostasis. It is also involved in the regulation of alveolar cell differentiation, survival and apoptosis. This pathway is very frequently mutated in breast cancer (42). The protein serine/threonine kinase Akt1 is required for the functional differentiation of the secretory epithelium and metabolic pathways that regulate milk synthesis (43). The ablation of Akt1 in transgenic glands interfered with the phosphorylation of Stat5a and delayed the alveolar differentiation during pregnancy and lactation (44). It has been also shown that Prl-mediated activation of Stat5 regulates the transcriptional activation of the Akt1 gene (45).

A downstream substrate of activated Akt includes the serine/threonine kinase mammalian target of rapamycin (mTOR). The mTOR pathway is also essential for growth and differentiation of mammary epithelial cells (46). Inhibition of mTOR activity impaired mammary epithelial cell proliferation, alveolar differentiation and milk protein synthesis. The effects of mTOR on proliferation and differentiation are mediated by the functions of the helix-loop-helix proteins Id1 and Id2. The mTOR activity has distinguishable functions in the proliferative and the differentiated state of mammary epithelial cells: it is a prerequisite for proliferation through the induction of Id1 and for differentiation-specific gene expression through the

induction of Id2. The relative strengths of these proliferation and differentiation signals reflected by the expression levels of the individual Id proteins are crucial to the functional life cycle of mammary epithelial cells. Id2 appears to control the proliferation and survival of alveolar cells during late pregnancy in response to the RANKL stimulation (47).

The recepor activator of nuclear factor kappa-B ligand (RANKL) directly induced proliferation mainly through the activation of the NF-kappaB pathway and the expression of Cyclin D1, a downstream target gene. Previous studies showed that RANKL expression is regulated by P or Prl signaling mediated by Stat5 (10). This suggests that Stat5 might affect cell proliferation through the NF-kappaB pathway. In addition, the expression of the Cyclin D1 gene is also directly regulated by Stat5.

The insulin-like growth factor 2 (Igf-2) mediates the role of Prl in luminal proliferation and induces directly Cyclin D1 expression (48). However, the enhanced expression of Igf-2 itself is not sufficient to induce alveologenesis. In hepatocytes and muscle, a transcription factor complex of glucocorticoid receptor (GR) and Stat5b tetramers induces the expression of Igf-1, a key regulator of postnatal body growth (49).

Elf5 is a member of the Ets transcription factor family, a regulator of the Prl signaling pathway and necessary to establish the secretory alveolar lineage during pregnancy (13).

2.3. Mammary epithelial differentiation and epigenetic regulation

MaSCs give rise to progenitors and finally to the luminal, ductal and alveolar and myoepithelial lineages. The mechanisms controlling the differentiation of the epithelial cell lineages involve networks of transcription factors (50) and stable programs of gene expression are embodied by epigenetic states. These epigenetic signals establish and maintain transcriptional states characteristic for particular stages of cellular differentiation. The epigenetic states are a reflection of the adaptation to environmental changes and specialized cell function in multicellular organisms. They maintain the activation and suppression of gene expression patterns through the persistent association of specific factors with particular chromatin domains (51). The epigenetic control is exerted by noncoding RNAs, DNA methylation and histone modifications (52, 53). These signals act in concert and orchestrate transcription from chromatin. The transmission of epigenetic information through cell division provides for the maintenance of cell identity in multicellular organisms.

Epigenetic regulation is most likely also a central determinant in the developmental stages of the mammary gland and reflected by the modification and organisation of chromatin domains. Distinct epigenetic characteristics have been detected in individual developmental stages of mammary epithelial cells (54). The chromatin structure at distal regulatory elements and promoters of milk protein genes, expressed in secretory alveolar cells, assumes a more open conformation when compared to cells in earlier stages of development. Chromatin changes can also be observed in the looping between regulatory elements and the attachment of chromatin to the nuclear matrix (55) and Stat5 activation is required to chromatin remodeling and the maintenance of mammary specific functions (56).

Distinct epigenetic characteristics have been found in human MaSCs and progenitor cells (57). The analysis of distinct subpopulations of mammary epithelial cells revealed discrete cell type and differentiation state specific DNA methylation patterns and gene expression profiles. $CD44^{+}$ cells were the most hypomethylated cells and expressed transcription factors with known stem cell functions. Epigenetically controlled transcription factors seem to play important roles in the regulation of mammary epithelial cell phenotypes, although the mechanisms with which they confer their effects on states of chromatin conformation and compaction are not well understood yet (58).

3. THE STAT FAMILY OF TRANSCRIPTION FACTORS AND STAT5 FUNCTIONS

The Stat proteins are a family of latent transcription factors that mediate the transmission of extra-cellular signals, like hormones, growth factors and cytokines, from transmembrane receptors to target gene promoters in the nucleus. They were originally discovered in the context of interferon dependent gene expression in human cells (59) and subsequently found to play essential roles in multiple cytokine signaling pathways. The seven distinct Stats range in size from 750 to 900 amino acids, share a common organization of their structural domains and a common mode of activation.

Stat5 controls diverse physiological processes in different organs, including the mammary gland, blood cells and hepatocytes. Stat5 activation by Prl led to the discovery of this transcription factor and the description of its basic mechanism of action, involving receptor mediated tyrosine phosphorylation, dimerization, translocation to the nucleus and transactivation of target genes, *e.g.* the beta-casein and whey acidic protein (WAP) genes, gap junction protein connexin 26 and suppressor of cytokine signaling (Socs2 and Socs3), cytokine inducible SH2-containing protein (Cish), Cyclin D1 and Elf5 (10, 60).

Stat5 activity is also required for the functions of many immune cells (61). Deregulated Stat5 activity is detrimental, and hyperactive Stat5 can cause cancer, myelo proliferative diseases, inflammation or autoimmunity. Hypoactive Stat5 can cause myeloid hypoplasia (anemia, thrombocytopenia), dwarfism, infertility, immunodeficiency or metabolic syndromes (62). For these reasons, Stat5 activity is tightly controlled, mainly through post-translational modifications such as glycosylation, ubiquitinylation, serine/threonine phosphorylation and tyrosine phosphorylation and by interacting proteins. The inappropriate activation of Stat5 tyrosine kinases can convert it into an oncogene, most thoroughly studied in leukemias and lymphomas, but also documented in liver

cancer, lung cancer, ovarian cancer, head and neck cancer, breast cancer and prostate cancer (63).

In addition to the well-established signaling pathway, non-canonical Jak/Stat functions have been discovered. In Drosophila melanogaster direct control of heterochromatin stability was observed. The unphosphorylated form of Stat92E, the ortholog of Stat5, is localized in the nucleus on heterochromatin and stabilizes it by binding to the heterochromatin protein 1 (HP1). Phosphorylation of Stat92E by Hop, the ortholog of Jak2, induces HP1 dispersal and causes heterochromatin instability. Genes localized in heterochromatin become thus accessible to phospho-Stat92E or other transcription factors (64).

Unphosphorylated Stats, up to now mainly exemplified by Stat3, can also regulate gene expression through the interaction with another signaling components, *e.g.* Stat3 and NF-kappaB (65). Additional non-canonical functions of Stat3 were found in tumorigenesis which required serine (S727), but not tyrosine phosphorylation. It has been shown that the mitochondrial localization of Stat3 supports the Ras-dependent malignant transformation and the loss of Stat3 led to a 50% reduction in cellular ATP levels (66). In spite of this transformation-specific function of Stat3, it could also be found in mitochondria of non-transformed cells and primary tissues, including the liver and heart. This mitochondrial Stat3 was shown to modulate respiration in mouse heart tissue and is required for optimal function of the electron transport chain and cellular homeostasis (67).

Another unconventional function of Stat5 is the activation of PI3K/Akt pathway. This function requires the interaction of tyrosine phosphorylated Stat5 with the scaffold protein Gab2 (68).

4. STAT5 FUNCTIONS IN MAMMARY GLAND DEVELOPMENT

Stat5 was originally identified as mammary-gland-specific nuclear factor (MGF) in the mouse mammary epithelial cell line, HC11 (69). The ortholog of MGF was cloned from sheep mammary gland (70) and was shown to mediate the transcriptional effects upon prolactin receptor (PrlR) activation (71). MGF was subsequently designated Stat5 (Stat5a, 94 kDa) due to its sequence homology with other members of the Stat family. A second isoform of Stat5 (Stat5b, 92 kDa) was discovered in the mouse mammary gland (72), with 95% sequence homology to Stat5a, but encoded on a separate gene. Further studies revealed that Stat5b mainly mediates the biological effects of GH in muscle and liver. The non-receptor tyrosine kinase 2 (Jak2) phosphorylates the conserved tyrosine residues, Y694 in Stat5a and Y699 in Stat5b, and causes their activation.

The latent form of Stat5a is constitutively expressed in mammary gland cells, but Stat5 is tyrosine phosphorylated and activated only during pregnancy and lactation. It controls the formation and terminal differentiation of the alveolar units. Stat5a activation is necessary for the establishment of luminal progenitor cells (60) and its activation is sufficient for the luminal alveolar cell fate decision (73).

Pituitary Prl is the major driver of alveologenesis during pregnancy (74). This peptide hormone binds to PrlR a member of the cytokine receptor superfamily (75), and activates multiple signaling pathways: Jak2/Stat5 (71), Ras/MAPK (76, 77), PI3K/Akt (78, 79) and Vav/Rac pathway (80). Despite the ability of the PrlR to activate diverse signaling cascades simultaneously, Stat5 activation seems to be the predominant signal. Mammary glands of mice lacking Stat5 show similar defects in alveologenesis as Prl and PrlR knockout mice. This suggests that Jak2/Stat5 pathway is the central signaling component activated by Prl hormone in the regulation of alveologenesis.

Other growth factors or hormones, *e.g.* epidermal growth factor (EGF), amphiregulin (AREG) and growth hormone (GH) can also activate Stat5a in mammary cells *in vitro* (81, 82). However, it is likely that *in vivo* GH and EGF activate Stat5 preferentially in the mammary stroma (83).

The extent and the duration of Stat5 activation is restricted by several negative regulators, like Socs1 and Socs2. They are direct Stat5 target genes and control Stat5 activation through negative feedback regulation of PrlR signaling. Mammary gland specific deletion of these negative regulators led to precocious activation of Stat5a and alveolar differentiation (84, 85). Stat5 activity is also regulated by membrane bound Caveolin1 (Cav1) and phosphatases like SH2 domain-containing phosphatase (SHP-2), that attenuates Jak2 kinase activity and dephosphorylates the activated Stat5.

Stat5 activation has a biphasic pattern. Its transient activation occurs very fast upon Prl stimulation, but is not sufficient for milk protein expression. The transient activation may play a role in mammary gland development in correlation with transient Prl secretion during estrus and in early pregnancy (56, 86). Sustained activation of Stat5 during lactation could be necessary for chromatin remodeling, and histone acetylation in the promoters of milk protein genes and the maintenance of the mammary-specific functions (56).

4.1. Stat5 transgenic mouse models

Transgenic mouse models have been used for the functional analyses of genes involved in the growth and differentiation of the mammary gland (10, 87, 88). Many of these studies were based on gene deletion procedures. Individual genes were disrupted in embryonic stem cells and mice were derived in which all somatic cells homogeneously carried the introduced genetic defect. Alternatively, gene disruption was carried out in a tissue specific manner, a procedure which limits the gene disruption to mammary epithelial cells (MECs) and cells of particular stages of differentiation. A summary of main advantages and disadvantages of these methods is shown in the Figure 2a.

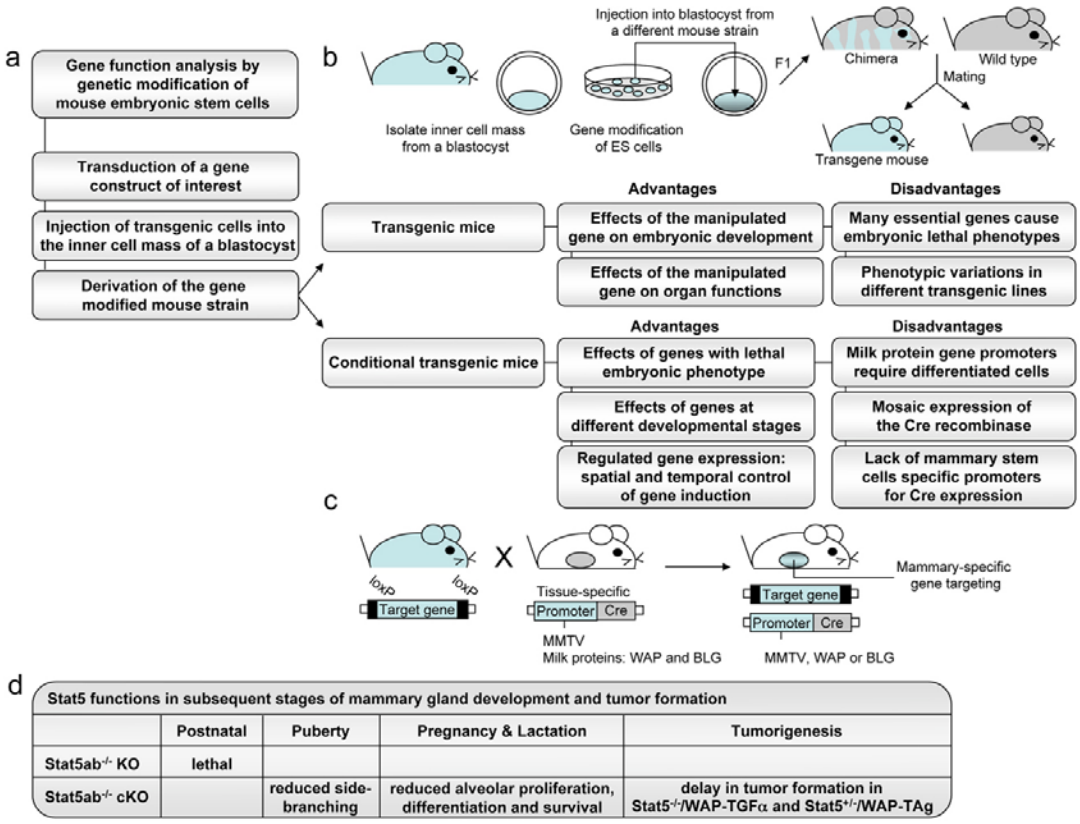


Figure 2. Gene function analysis in the mammary gland by the generation of different transgenic mouse models. a) The flowchart shows the main advantages and disadvantages of different knockout transgenic mouse models for the gene function analysis in the mammary gland. Germline inactivation of selected genes represents valuable tools for the study of gene functions in the whole body including mammary epithelial and stromal components during the pre- and postnatal development. Manipulation of essential genes is frequently lethal or exhibits phenotypes in other tissues. To circumvent this limitation, mammary specific knockout mice were generated. In these animals, the expression of Cre recombinase is driven by the promoter of mouse mammary tumor virus (MMTV) or of milk protein genes like whey acidic protein (WAP) and beta-lactoglobulin (BLG). The expression of Cre recombinase in mammary glands of these transgenic mice is usually not uniform and these promoters are not specific for mammary stem cells. The milk protein promoters used are only induced in differentiated secretory alveolar cells. b and c) Schematic representation of usual techniques for the generation of transgenic and conditional knockout mice. b) Embryonic stem (ES) cells are harvested from the inner cell mass (ICM) of mouse blastocysts. They can be cultured and genetically modified *e.g.* with the viral transfer vectors without losing their pluripotency. ES cell clones with the site-specific recombination will then be selected and injected into the blastocyst-stage embryos from a different mouse strain. These genetically modified ES cells divide and become part of the embryo, which results in the generation of chimeric animals. In case that the ES cells contribute to the germ cells in chimeric mice, the recombinant genotype can be transmitted into the offspring. c) Mammary specific inactivation of the gene of interest can be achieved by means of a Cre recombinase enzyme (under the control of a mammary-specific promoter) that deletes the DNA fragment located between the two loxP-recombinase-specific sites (Cre-loxP site-specific recombination system). d) Distinct functions of Stat5 in the mammary gland development and in tumor formation were identified using Stat5ab$^{-/-}$ knockout (KO) and conditional knockout (cKO) mouse models.

Since genes may fulfill multiple and diverse functions, the inactivation of a gene in all cells and tissues of the body can be instructive. Information can be gained about the function of a gene involved in embryonic and adult development and about effects on the proper function of cells derived from all three germ layers (Figure 2b). This approach, however, is limited by the observation that genes are often essential in embryonic development and their inactivation in early embryonic cells arrests development and causes embryonic lethality. In addition, they might exhibits phenotypes in organs or tissues which indirectly contribute to the phenotype of other organs. This is especially evident for organs as the mammary gland which depend upon the maturation of endocrine organs and the secretion of circulating hormones.

The problems arising from embryonic lethality of gene disruption can be circumvented and the study of genes can be restricted to their functions in particular cell types through the generation of conditional knockout mice

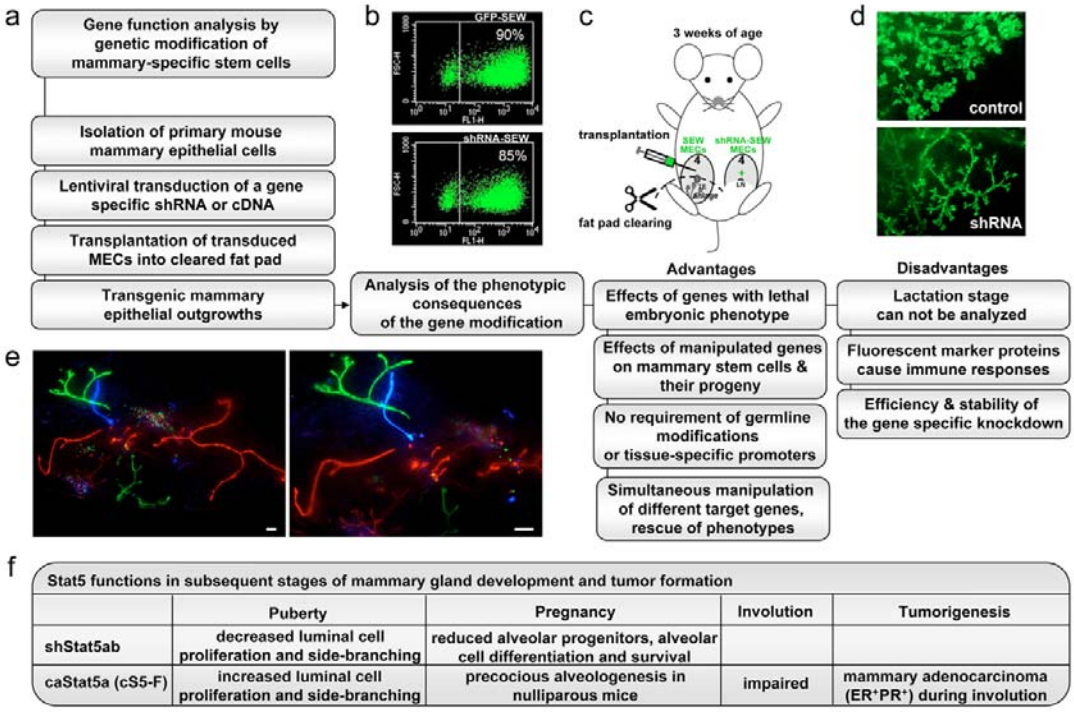
(Figure 2c). This approach is dependent upon the expression of a Cre recombinase under the control of a cell type and differentiation stage specific promoter and the subsequent elimination of a floxed target gene. It has been successfully applied for the investigation of gene functions in the mammary gland. However, also this technology still suffers from practical limitations: 1) promoter constructs with limited cell type and stage specific activity were used for the expression of the Cre recombinase; 2) these promoter constructs often require hormonal signals for their regulation (89); 3) the expression of the Cre recombinase might result in phenotypic consequences (90); 4) the Cre expression and the recombination of the floxed target genes in the mammary tissue are usually not entirely uniform and chimeric situations are encountered and 5) the promoters used in these studies are not active in MaSCs; *e.g.* the promoters for milk protein genes, like WAP and beta-lactoglobulin (BLG), are only induced in secretory alveolar cells at late stages of differentiation (91, 92). Reports about the expression and specificity of the mouse mammary tumor virus (MMTV) promoter in MaSCs are controversial and a few papers could show its activity in MaSCs (60).

Many molecular components of the Jak/Stat pathway have been investigated in transgenic mouse models. Embryonic lethality was observed for Jak1, Jak2, Stat3 and Stat5a/b, but not in the absence of Stat1, Stat2, Stat4, Stat5a, Stat5b and Stat6. Stat3 knockout mice die at a very early stage of embryonic development, prior to mammary bud formation. Because Stat3 is a key transcriptional determinant of embryonic stem cell self-renewal and pluripotency (93), it will be interesting to determine its role for the establishment of MaSCs in prenatal development and the maintenance of the MaSCs pool during involution.

The disruption of Stat5 dependent cytokine signaling results in abnormalities in cells of the immune and hematopoietic systems, and also in hepatocytes, mammary and prostate epithelial cells (49, 94). These insights were gained from gene inactivation studies in mice and spontaneously occurring Stat5b mutations in humans (95-97). Transgenic mice lacking either the Stat5a (98) or Stat5b (99) gene were derived, they are viable and display distinct defects. Stat5a deficient mice developed normally, but females failed to lactate due to impaired alveologenesis and males exhibited a defective prostate epithelium. Stat5b gene disruption affected body growth, liver gene expression, lactation, reproduction, fat deposition, hair growth and defects in NK cell activity. Stunted body growth was observed only in male animals and was due to the loss of responsiveness to GH pulses (GH pulse-resistant), which regulates sexually dimorphic liver gene expression (99). $Stat5b^{-/-}$ females consistently aborted because of a strong decline of serum P.

The first knockout mice that lack both Stat5 genes, Stat5a and Stat5b (collectively called Stat5), were generated (100) and largely confirmed the observations made with Stat5a and Stat5b single knockout mice. However, the loss of additional functions associated with growth hormone or Prl signaling were found. These mice displayed profound defects in T cell lineages, specifically in the proliferation of peripheral T cells. Initially no effect on erythropoietin (EPO) function and the production of red blood cells was observed and the mice survived until adulthood. Another group however, obtained different results with the same knockout mouse strain. They claimed that in adult mice with normal steady-state hematocrit, Stat5 expression was essential for the high erythropoietic rate during fetal development (101). Stat5 knockout embryos were severely anemic and exhibited lower numbers of erythroid progenitors, less response to EPO and higher levels of apoptosis. In addition, female mice were infertile because of defects in the corpora lutea and could not directly be used for mammary gland differentiation studies. Transplantation studies of Stat5-deficient mammary glands into cleared fat pads of wild type mice and mammary gland analysis during pregnancy showed impaired alveologenesis similar to Stat5a knockout mice. In spite of abnormalities in the hematopoietic system in these transgenic mice, the resulting phenotypes were not as severe as observed in animals with cytokines receptor mutantions. Subsequently, it was proposed that these knockout mice express N-terminally truncated Stat5a and Stat5b proteins ($Stat5^{\Delta N}$) which are able to form dimmers, but not tetramers (49). It was suggested that these mice express truncated Stat5 proteins which act as hypomorphs and still activate some target genes.

These controversies were finally resolved by experiments carried out in the laboratory of Lothar Hennighausen. These investigators generated Stat5 double knockout mice in which the entire 110-kb Stat5a/b locus was deleted by a single recombination event (102). The resulting mice exhibited early postnatal lethality (Figure 2d). They die shortly after birth due to severe anemia associated with reduced transferrin receptor (Tfr1) gene expression (103). The early lethality precludes that these mice can be used to study the effect of Stat5 loss of function on mammary gland development in adult virgin mice and during pregnancy. For this purpose conditional knockout mice had to be generated (102). The mammary specific deletion of the Stat5a/b 110-kb locus, flanked by loxP sites, was performed using the expression of Cre recombinase under the MMTV or WAP promoters. Stat5 deletion prior to puberty in Stat5fl/fl/MMTV-Cre expressing knockout mice did not affect ductal outgrowth and primary and secondary branching in mature mice. However, gene deletion during late pregnancy in Stat5fl/fl/WAP-Cre knockout mice resulted in impaired alveologenesis (Figure 2d). These studies, however, were also hampered by the sequential and mosaic expression of the Cre recombinase in the cells of the transgenic glands. This resulted in relatively few cells which lacked Stat5 at parturition, a time point when WAP expression is not yet maximal. The authors found explanations for the presence of the relatively small number of Stat5-null cells in Stat5fl/fl/WAP-Cre glands and pointed out: 1) the loss of Stat5 resulted in an inability to maintain the differentiation stage and caused elimination of these Stat5-null cells by apoptosis, and 2) the cells that failed to undergo Cre-mediated Stat5 deletion had growth and survival advantages over the Stat5-null cells.



Stat5 functions in subsequent stages of mammary gland development and tumor formation

	Puberty	Pregnancy	Involution	Tumorigenesis
shStat5ab	decreased luminal cell proliferation and side-branching	reduced alveolar progenitors, alveolar cell differentiation and survival		
caStat5a (cS5-F)	increased luminal cell proliferation and side-branching	precocious alveologenesis in nulliparous mice	impaired	mammary adenocarcinoma (ER^+PR^+) during involution

Figure 3. Gene function analysis using reconstitution of the mouse mammary epithelium with genetically modified stem cells. a) The flowchart shows the main advantages and some limitations of the functional gene analysis through the cleared fat pad transplantation of genetically manipulated mammary stem cells (MaSCs). This method facilitates functional analysis of individual genes or even a combination of genes in a less laborious and time-consuming way. This provides an alternative approach to transgenic mouse models, which require germline modifications or tissue-specific promoters. The expression of candidate genes can be inactivated or enhanced in primary mammary epithelial cells (MECs) using lentiviral transfer vectors (*e.g.* self-inactivating SEW and SiEW vectors (73)) expressing either specific shRNAs or cDNA sequences. This method enables to rescue phenotypes induced by the loss or gain of function of target genes using the expression of downstream effectors or specific shRNAs. This procedure is suited to analyze specific genes playing crucial roles in postnatal development of the gland, particularly genes with lethal phenotypes in knockout mice. The lactation stage cannot be fully investigated using this procedure, because the transgenic ducts do not connect to the nipple and undergo involution shortly after parturition due to milk stasis. b) Gene modification of primary MEC populations and its rare stem cell fraction can be achieved *ex vivo* by efficient lentiviral transduction under the adherent condition. c and d) Transplantation of gene-modified MECs and control cells in cleared fad pads of a recipient mouse allows to monitor the effects of manipulations on the stem cells activity, lineage commitment and differentiation capacity. e) Interactions and competitions between individually transduced MECs can be analysed. Transplantation of a mixture of MECs expressing different fluorescent markers (Cerulean, Venus and mCherry) resulted in transgenic outgrowths originating from individual transduced stem cells, which grow in a territorially restricted fashion. Scale bar is 500 µm. f) Distinct functions of Stat5 in mammary gland development and in tumor formation were characterized using loss and gain of Stat5 functions in MaSCs by the expression of Stat5 specific shRNAs and a constitutively activated Stat5a mutant (cS5-F).

The whole mount analysis of PrlR- and Stat5-null (from $Stat5^{\Delta N}$ knockout mice) mammary epithelia transplanted into wild type hosts revealed no obvious side-branching phenotype in virgin mice. A more pronounced phenotype was observed during pregnancy. In particular, alveolar differentiation in the PrlR-null glands was stronger inhibited than in the Stat5-null glands, probably due to the activation of additional signaling pathways by Prl (104).

4.2. The analysis of gene functions using reconstitution of the mouse mammary epithelium with genetically modified stem cells

Mouse mammary stem cells are present within the tissue of mature mammary glands and can be functionally identified through their ability to reconstitute a glandular tree upon transplantation into cleared fat pads. Single cells were shown to be able to regenerate mammary epithelium with developmental potential. Mammary epithelial reconstitution can also be carried out with stem cell populations which have been genetically modified *ex vivo*. These manipulated MaSCs and progenitors provide valuable tools to study the functions of individual genes in differentiation and transformation of the mammary gland. The main advantages and disadvantages of these methods are shown in the Figure 3a. This procedure is suited to analyze specific genes which are important for the postnatal development of the gland when reproductive and lactogenic hormones regulate puberty and pregnancy. But also the

transgenic approach based on the transplantation of modified stem cells has limitations. The lactation stage cannot be fully investigated using this procedure, because the transgenic ducts emerging from the exogenously supplied MaSCs, do not connect to the nipple and milk made in the secretory alveolar cells can not be removed by suckling. The cells undergo involution shortly after parturition due to milk stasis.

Since there is only a small number of stem cells present in the unfractionated population of the mammary epithelial cells, efficient gene transfer is required to affect the MaSC compartment. This can be achieved with lentiviral gene transduction vectors. Primary MECs can be infected under adherent conditions (73) (Figure 3b) and a very high percentage of the cells can be stably modified. These cells can then be transferred into cleared fat pads which provide a natural microenvironment (niche) for the selection of gene-modified stem cells (Figure 3c). Only stem cells will, by definition, grow out and yield the cell types able to reconstitute the ductal tree. If the exogenously supplied stem cells have been marked with a gene encoding a fluorescent protein and the modified stem cells have been introduced into a wild type mouse fat pad, the progeny of the transplanted stem cells can be easily identified. Ducts originating from GFP expressing stem cells are shown in Figure 3d. Functional genomics studies can make use of lentiviral vectors encoding a fluorescent protein which serves as indicator for the transduction, transplantation and reconstitution efficiencies, and a cDNA or a shRNA which elicit gain or loss of function phenotypes (Figure 3d).

Several parameters limit the frequency with which the virally infected transgenic stem cells form ductal structures. The efficiency of the lentiviral transduction into the primary MECs is one of them. If infected and uninfected stem cells simultaneously contribute to the reconstituted ducts, chimeric structures will result. Another parameter limiting the efficiency of reconstitution is immunogenicity. The expression of *e.g.* the fluorescent protein GFP elicits immunoreactions in wild type recipient mice and limits the efficiency of the engraftment. This problem can be circumvented by the introduction of the modified stem cells into immunodeficient mice.

The mouse model based on genetic modification of stem cells combined with transplantation of the genetically modified cells into cleared fat pads provides a number of additional experimental benefits. Corresponding control cells, *e.g.* transduced with a control vector lacking a particular cDNA or expressing a scrambled shRNA sequence, can be introduced into the contralateral inguinal fat pads of the recipient mice for direct comparisons. The functional properties of the transduced genes and the consequences of their expression for the hierarchy and phenotypes of the mammary cell types can be investigated, *e.g.* 1) the self-renewal and the repopulation potential of MaSCs, 2) the cell fate decisions in progeny of MaSCs, 3) The proliferation and survival of luminal and alveolar progenitor cells, 4) the differentiation and survival of secretory luminal cells and 5) the induction of mammary neoplasia.

The transduction of stem cells with distinct fluorescent proteins and the reconstitution of the ductal tree with a mixture of these cells provides additional information on the number of stem cells involved and their interactions. The stem cells encoding different fluorescent proteins showed that reconstitution is oligoclonal, *i.e.* a relatively small number of stem cells are required to form a ductal tree. The individual ducts were composed of cells of a single color indicating their clonal origin (Figure 3e). Since the stem cells can be infected with more than one virus 1) interactions between individually transduced cells, 2) the functional analysis of genes and genetic networks using simultaneous transductions and 3) the rescue of phenotypes induced by loss or gain of function studies using the expression of downstream effectors or specific shRNAs can be addressed.

Transgenic mouse models, in which one (98, 99) or both Stat5 isoforms have been altered, (100, 102) exist. Nevertheless, the consequences of Stat5 manipulation in MaSCs and arising progenitors and differentiated cells could not be precisely investigated in these animals. One obstacle has been the lack of stem cell specific promoters to express the Cre recombinase in these cells. The genetic modification of MaSCs was therefore most useful to gain additional information about Stat5 functions in the mammary epithelium (Figure 3f). Downregulation of both Stat5 isoforms in MaSCs does not influence their engraftment capacity and the morphology and structure of ductal outgrowths. However, these transgenic grafts show much less tertiary branching in virgin mice which is important for the alveologenesis during pregnancy (73). Similar to the phenotype observed in Stat5 conditional transgenic mice, grafts expressing shStat5 show impaired alveolar development.

Conversely, the introduction of a constitutively active mutant of mouse Stat5a (cS5-F, S710F) into MaSCs and their transplantation, led to glands in which the Stat5 variant fully replaced the function of lactogenic hormones and caused hyperproliferation of luminal cells, thickening of the ducts and precocious development of functional alveoli in virgin animals. Characterization of the different cell populations present in these transgenic grafts indicated that Stat5 activity regulates proliferation and/or survival of luminal progenitor cells and is sufficient for the emergence of mature alveolar cells from these progenitors. The persistent activation of Stat5 during the involution stage prevented apoptosis of terminally differentiated mammary secretory cells and induced tumorigenic transformation post-lactationally (73).

The comparison of these results with the existing Stat5 transgenic mouse models certified the utility of the method and revealed new insights into Stat5 roles in mammary gland development and tumorigenesis.

5. STAT5 INVOLVEMENT IN MAMMARY TUMOR FORMATION

Breast cancer is thought to originate from cancer stem cells (CSCs) emerging from transformed MaSCs or

progenitor cells with acquired self-renewal capacity (105). CSCs share features with their normal counterparts; these biochemical and functional properties can be utilized for their enrichment. Markers include such as the increased activity of aldehyde dehydrogenase 1 (ALDH1) (106), lower levels of reactive oxygen species (ROS) (107), the expression of the transmembrane glycoprotein CD44 (108) and functional properties the deficiency in gap junctional intercellular communication (109, 110), anchorage-independent growth and the ability to survive in a hypoxic microenvironment (111).

Cancer cell growth frequently becomes independent from exogenous cytokine and growth factors (112). This can be due to the autocrine action of factors secreted by tumor cells or mutations in the genes encoding the receptors for such factors. Consequently, the persistent activation of signaling molecules such as receptors, kinases and transcription factors, *e.g.* Stat proteins (113, 114), can be observed. Inappropriately activated Stat5 can assume the role of an oncogene. Activated Stat5 has been observed in different human cancers. It affects tumor cell proliferation and survival. Dysregulation of the physiological Stat5 activation patterns and its persistent activation is found in a variety of solid tumors, including breast and prostate cancer (94, 115). In these tumors Stat5 activity is usually associated with GH and Prl expression. In hematopoietic malignancies activated Stat5 promotes self-renewal of CSCs.

A high percentage of human breast cancers express phosphorylated Stat5. A stratification of the tumors showed that Stat5 activation is usually associated with a favorable prognosis for the patient (94). This might reflect the dual role of Stat5. Stat5 is an important differentiation factor in mammary epithelial cells and promotes the formation of secretory alveoli and the induction of milk protein gene expression. At the same time it enhances the proliferation of luminal epithelial cells and protects these cells against the induction of apoptosis. It is possible, that even in tumor tissue, Stat5 retains some of its differentiation potential and thereby limits the aggressiveness of the tumor cells. This is reminiscent of the dichotomy of the estrogen receptor function (116).

Activating events affecting the status of Stat5 are usually found in components acting upstream in the signaling pathway. They comprise mainly consequences of mutation or amplification in growth factor receptors and tyrosine kinases especially Jak2. However, downstream negative regulators can also be affected, resulting in a prolonged and enhanced cytokine signal (117). Additionally, novel mechanisms and nuclear functions for these tyrosine kinases are coming into focus. In the nucleus, Jak2 can directly phosphorylate Tyr 41 of histone H3 (H3Y41) and exclude heterochromatin protein 1 alpha (HP1-alpha) from chromatin (118). The displacement of HP1-alpha by constitutively activated Jak2 increases the gene expression, mitotic recombination and genetic instability. The consequences for Stat5 transactivation by this nuclear function of Jak2 are not known yet, but is it conceivable, that it could affect accessibility and recruitment to Stat5 DNA binding sites.

5.1. Stat5 transgenic mouse models for mammary tumor studies

In order to study the function of persistent activation of Stat5 in the transformation process independently of upstream activation events, different constitutively activated variants of Stat5 protein were generated (119-124). The first construct was a chimeric protein made by fusion of the kinase domain of Jak2 to the carboxyl terminus of Stat5a. This constitutively activated Stat5 (Stat5ca) fusion protein had self-activating properties, *i.e.*, the kinase domain was able to phosphorylate tyrosine residue 694 causing dimerization and DNA binding in a cytokine-independent manner (125). In order to obtain a stronger transactivation potential, the Stat5 transactivation domain was exchanged in the chimeric protein against the transactivation domain of Stat6. This construct was used for the generation of transgenic mice. The consequences of overactivation of Stat5 on mammary gland development and tumorigenesis were studied (119).

Other constitutively active forms of STAT5 were generated by PCR-driven random mutagenesis and identified using retrovirus-mediated expression screening. Most of these mutants were tested *in vitro* and *in vivo* mainly in the hematopoietic system (123, 126). The Stat5a1*6 (cS5RF) and Stat5a1*7 mutants contain two amino acid substitutions. These mutations are located upstream of the DNA binding domain or in the transactivation domain (S710F) and are transcriptionally active in the absence of cytokine signals (121). Moriggl *et al.* constructed the cS5F mutant, a variant with only a single point mutation (S710F), and investigated the role of persistent Stat5a activation in leukemia induction. The cS5F mutants form tetramers and are functionally active. Transplantation of cS5F-transduced bone marrow cells into recipient mice led to tumor formation. Reminiscent of the situation in human leukemic samples in which Stat5 displays a strong tetramer formation, the cS5F mutant caused the development of multilineage leukemias in mice (123).

The involvement of Stat5 is not restricted to leukemia formation, but has also been documented in tumor models of the rodent mammary gland (94, 114). Stat5a has been shown to be induced in rat mammary gland tumors induced by chemical carcinogens (127) and the nuclear presence of Stat5a was correlated with higher-grade carcinomas. Stat5a activation was found in cells proliferating intraductally and in ductal carcinomas *in situ* (DCIS). The effects of activated Stat5a in mouse mammary gland tumorigenesis has also been investigated. WAP-TGF-alpha transgenic mice develop mammary tumors with a distinct frequency and latency. When these mice were crossed with $Stat5a^{-/-}$ mice, the resulting $Stat5a^{-/-}$/WAP-TGF-alpha transgenic mice showed a delayed in the induction of tumor formation (128). Stat5 enhances the progression of TGF-alpha-mediated mammary tumorigenesis and might aid the survival of tumor cells. In a second tumor model, SV40-T antigen transgenic mice (WAP-TAg) were crossed with hemizygous Stat5a mice.

Stat5a$^{+/-}$/WAP-TAg mice also showed a delay in tumor formation, decrease in tumor size and an increase of the apoptotic index in the cells of the developing adenocarcinomas (129).

A tissue specific transgenic mouse model has been derived in which the persistent Stat5 activation could be studied in the mammary gland. In this model, the expression of the constitutively active Stat5 variant, Stat5ca, is regulated by the BLG milk protein gene promoter (119) and cytokine independent Stat5 activation persists upon the emergence of milk protein producing secretory alveolar cells. About 10% of the multiparous BLG-Stat5ca transgenic mice developed highly differentiated adenocarcinomas after a latency of 8 to 12 months (130, 131).

Although this mouse model provides the first experimental evidence that constitutively active Stat5a can promote the occurrence of sporadic mammary cancers in mice, there are several limitations to this transgenic model: 1) the kinase domain of Jak2, present in the artificial Stat5ca chimeric protein, could contribute to the transformation potential through the phosphorylation of other substrates; 2) the BLG-Stat5ca transgene is activated only in differentiated secretory alveolar cells, which are largely eliminated during involution. Transformation is therefore possibly only an exceptional event in a small number of cells and this might explain the delayed onset of tumor formation; 3) there are reports that FVB mouse strain used in this model can develop mammary hyperplasia due to pituitary abnormalities in aged mice (114, 132-134). However, Iavnilovitch and colleagues did not observe any tumor formation in the control mice. These points make it important to substantiate these findings independently in additional mouse models.

5.2. Persistent stat5 activation in mammary stem cells causes post-lactational tumor formation

The conditional expression of oncogenes in a tissue specific manner has been of great value to gain insights into the function of oncogenes in the mammary gland and in mammary tumor formation (135) and studies which investigated the requirement of persistent oncogene expression for the maintenance of tumors led to the concept of oncogene addiction (111, 136). These experiments are usually complex, lengthy and labor intensive and require the introduction of specific mutations in the germ line, the expression of the Cre recombinase under the control of a tissue specific promoter and the derivation of double transgenic mouse lines. These requirements can be circumvented by a experimental protocol based on the genetic modification of adult stem cells and the reconstitution of organ specific functions through transplantation into depleted mice. This strategy, however, is limited to only a few organs. The hematopoietic system, *e.g.*, can be reconstituted by exogenously provided stem cells upon irradiation. Conceptually similar, it is possible to reconstitute the mammary epithelium by the transplantation of exogenously supplied stem cells. The genetic manipulation of the stem cells prior to their transplantation provides a powerful model for the study of growth, differentiation and the control of organogenesis in this organ. The deregulation of genes which cause aberrations in proliferation and differentiation programs are most relevant for progress in breast cancer research.

We have applied this experimental approach to the study of Stat5. Several transgenic mouse models have previously suggested that Prl signaling and Stat5 activation can contribute to mammary tumor formation (114). In addition, immunohistological analysis has shown that Stat5a is activated in a high percentage of breast tumors (115). Our observations are consistent with these findings and complement them with mechanistic insights. The introduction of persistently activated Stat5 variant, cS5-F (S710F), into mammary stem cells and their transplantation into cleared fat pads initially leads to the enhanced proliferation of luminal epithelial cells and precocious alveolar differentiation in virgin mice. During the involution stage it prevents apoptosis of terminally differentiated secretory cells and finally induces the formation adenocarcinomas (73). Tumorigenic transformation occurs only post-lactationally. When compared to the BLG-Stat5ca tumors, interesting differences can be identified: 1) cS5-F tumors were induced with short latencies usually subsequent to pregnancy and lactation and 2) cS5-F mutant was expressed in MaSCs, progenitor cells and all subsequent epithelial progeny.

The cS5-F-induced tumors occurred at a stage when Stat5 activity is normally downregulated, *i.e.* after the cessation of suckling and the induction of apoptosis. The persistent activity of Stat5 initially protected the fully differentiated alveolar cells from apoptosis and cell survival became independent from the continued secretion of Prl. Subsequently, malignant transformation was observed during the involution phase. These tumors were classified as mammary adenocarcinomas and could be serially transplanted into secondary virgin hosts. The primary and secondary tumors were highly proliferative, expressed activated Stat5 and Stat3. They contained a fraction of Lin$^-$CD24^{low}CD44$^+$ cells, markers present in CSCs. Microarray analysis of cS5-F-expressing ducts and tumors revealed the enhanced expression of Stat5 target genes. Previously identified target genes and many novel genes, regulated downstream of activated Stat5, were found. These genes are candidates with putative roles in gland development or in malignant transformation.

5.3. CS5-F-Induced tumors serve as a novel ER$^+$ mammary tumor model

Different experimental models have been developed to investigate the multistage process of breast cancer initiation and progression and its histopathological features, genetic variability and prognostic outcomes (89, 137). These include 1) breast cancer cell lines, which can be studied *in vitro* under 2D and 3D culture conditions to address questions concerning proliferation, apoptosis and migration of transformed cells, 2) xenografts of cell lines or clinical isolates of human tumors, which allow the investigation of systemically acting factors and tumor-stromal interactions in *in vivo* environments, and 3) genetically engineered mouse models of breast cancer,

which provide valuable models for studying the biology and pathogenesis of breast cancer (89). None of these models alone can recapitulate all aspects of breast cancer due to the complexity and heterogeneity of the disease. In addition, most tumors from genetically engineered mice are hormone independent and do not resemble the ER^+ luminal subtypes most frequently encountered in human patients.

The tumors resulting from the persistent expression of cS5-F in mammary stem cells provides an *in vivo* model for the investigation of ER^+PR^+ in mice. Previous studies have shown that Stat5 is active in human breast cancer cell lines and that Stat5 activation is an important contributor to tumor cell survival. Dominant negative variants of Stat5 were able to induce apoptosis, *e.g.* in ER^+ T47D breast cancer cells (129, 138). The expression, tyrosine phosphorylation status and nuclear localization of Stat5 in primary human breast cancers were studied in 83 randomly selected primary human breast adenocarcinomas (115). Immuno-histochemistry, immuno-precipitation and Western blotting techniques were used to show Stat5a was localized in the nucleus and tyrosine phosphorylated in a high portion of the examined tumors (76%). In a second study, the expression of Stat3 and Stat5 in 517 human breast cancer tissues was investigated (139). In these tumors, Stat5 activation was significantly correlated with histological grade, ER and PR expression. Patients with Stat5 positive tumors (ER^+) had increased overall survival due to the better response to endocrine therapy (139).

In normal mammary glands, about 25 - 30% of ductal luminal cells express ER-alpha (140). These cells are also mainly PR and PrlR positive. FACS analysis according to the expression of CD24 and stem cell antigen-1 (Sca-1) defined three cell lineages in isolated Lin^- epithelial cells from virgin glands, namely the basal/myoepithelial, luminal epithelial ER^- cells ($Lin^-CD24^{+/high}Sca\text{-}1^-$) and luminal epithelial ER^+ ($Lin^-CD24^+/highSca\text{-}1^+$) (141). The proliferating cells in the mature glands are ER-alpha negative cells (140) and their proliferation is controlled by paracrine growth factors such as AREG, Igf-2, wnt4 and RANKL produced by ER-alpha positive cells (142). These paracrine mechanism may switch to an autocrine loop in early breast cancer progression. This allows the ER^+ tumor cells to proliferate, possibly through the downregulation of TGF-beta pathway. Many ER^+ mammary tumors have a high proportion of dividing cells that are ER-alpha positive (143).

Recently it has been shown that steroid hormone signaling controls the number and activity of mammary stem cells (32, 33). This number increases by about 11-fold at mid pregnancy, when the serum levels of P are at their highest. However, these stem cells have lower self-renewal capacity than dormant stem cells scattering throughout the ductal epithelium in virgin mice (33). The appearance of MaSCs during pregnancy was first suggested using parity-induced stem cells (PI-MaSCs) that did not undergo apoptosis and served as alveolar progenitors during subsequent pregnancies (144). Interestingly, PI-MaSCs facilitate tumor formation in multiparous females overexpressing Her2/neu (145). Stat5 functions on the regulation and transformation of these cells may explain its oncogenic effects in tumor initiation.

Another interesting property of the cS5-F induced tumor cells is the simultaneous activation of Stat5 and Stat3. Although both factors recognize similar DNA response elements and share target genes involved in the regulation of proliferation and apoptosis, they are characterized by distinct biological functions. In the mammary gland, Stat5 has been mainly associated with luminal cell proliferation and differentiation as well as with prevention of apoptosis. Stat3 seems to be required for the initiation of apoptosis. The deinduction of Stat5 at the end of lactation coincides the induction of Stat3 and beginning of the involution phase. Stat3, however, has been recognized as a potent oncogene in many tumors and clearly can contribute to cellular transformation (146). The analysis of reciprocal effects of Stat5 and Stat3 in human breast cancer shows that primary tumors with constitutive phosphorylation of both Stats were more differentiated than those with Stat3 activation alone. These tumors display more favorable prognostic characteristics and show increased sensitivity to chemotherapeutic drugs (147). The pro-inflammatory tissue environments during involution phase might also promote in tumorigenesis in transgenic grafts expressing persistent activated Stat5, cS5-F. There is some evidence that mammary gland remodeling during involution can promote the pregnancy associated breast cancer and metastasis (148).

RANKL was found as an important paracrine effector of P-induced MaSC expansion during pregnancy (32). RANKL mediates both P and Prl action and its activation results in precocious ductal side-branching and alveologenesis in virgin mice (149). Prolonged RANKL exposure in aged transgenic mice resulted in limited mammary epithelial hyperplasia and palpable mammary tumors (149). The expression of the RANKL gene is under the control of PRL and mediated by Jak2 and Stat5a. Further analyses will be necessary to determine the activation status and function of NF-kappaB in ER^+ breast cancer and to understand potential interactions with Stat5.

6. CONCLUSIONS AND PERSPECTIVES

Functional gene analysis based on the reconstitution of the mouse mammary epithelium with genetically modified stem cells yields new insights and complements the conventional transgenic methods. It allows the investigation of effects of genetic manipulations of stem cells, progenitor cells and differentiated cells and constitutes an important advance in the study of gene function in the mammary gland. It can be adapted to all genes which are not necessarily required for engraftment of the stem cells and formation of primary ducts and accelerates and facilitates functional genetics, tumor modeling and drug development.

Mouse mammary tumors induced by the expression of the cS5-F variant are ER^+PR^+ and mimic the luminal A breast cancer subtype. Estrogen is the major

regulator of postnatal mammary gland development. It exerts its effects mainly through ER-alpha expressed in the mammary stroma and epithelium. More than half of all diagnosed breast cancers express ER-alpha and around 70% of these tumors respond to anti-estrogen therapy (150). Unfortunately, ER^+ tumors can also fail to respond or become resistant to the conventional endocrine therapies. In order to understand the mechanisms that underlie this resistance and to develop strategies for overcoming or bypassing it, there is a need for ER^+ mammary tumor models. Due to the low circulating estrogen level in mice, most tumors from genetically engineered mice are hormone independent. The cS5-F mouse tumor model provides the possibility to study distinct functions of activated Stat5 in subsequent stages of mammary tumor initiation, growth and progression as well as the responsiveness to antiestrogens (tamoxifen) and aromatase inhibitors. The inhibition of Stat5 in these tumors will shed light on its role in tumor maintenance and its effects on invasiveness.

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Abbreviations: MaSCs: mammary stem cells; MECs: mammary epithelial cells; MPCs: multipotent progenitor cells; E: estrogen; P: progesterone; ER: estrogen receptor; PR: progesterone receptor; Prl: prolactin; Stat5a: signal transducer and activator of transcription 5A; PML: promyelocytic leukemia protein; p63: transformation related protein 63; APLs: acute promyelocytic leukemias; FOXA1: forkhead box protein A1; C/EBP-beta: CCAAT/enhancer binding protein; Id2: inhibitor of DNA binding 2; NF-kappaB: nuclear factor kappa-B; Elf5: E74-like factor 5; PI3K: phosphatidylinositol 3-kinase; mTOR: mammalian target of rapamycin; RANKL: receptor activator of nuclear factor kappa-B ligand; Igf-2: insulin-like growth factor 2; WAP: whey acidic protein; Socs2: suppressor of cytokine signaling 2; Cish: cytokine inducible SH2-containing protein; HP1: heterochromatin protein 1; MGF: mammary-gland-specific nuclear factor; PrlR: prolactin receptor; Jak2: tyrosine kinase 2; EGF: epidermal growth factor, AREG: amphiregulin; GH: growth hormone; Cav1: Caveolin1; SHP-2: phosphatases like SH2 domain-containing phosphatase; BLG: beta-lactoglobulin; MMTV: mouse mammary tumor virus; EPO: erythropoietin; Stat5-deltaN: N-terminally truncated Stat5a and Stat5b proteins; Tfr1: transferrin receptor; CSCs: cancer stem cells; ALDH1: aldehyde dehydrogenase 1; ROS: reactive oxygen species; Stat5ca: constitutively activated Stat5; cS5F: constitutively activated Stat5a with a single point mutation (S710F); DCIS: ductal carcinomas *in situ*; stem cell antigen-1 (Sca-1); PI-MaSCs: parity-induced stem cells; Lin^-: endothelial lineage marker negative; CD24: heat stable antigen; CD29: integrin-beta1; CD49f: integrin-alpha6; CD61: integrin-beta3; PL: placental lactogen; Gata3: GATA binding protein 3; Shh: sonic hedgehog signaling; ICM: inner cell mass; KO: knockout mouse; cKO: conditional knockout mouse

Key Words: Mammary Stem Cells, Mammary Lineage Commitment, Organ Reconstitution, Mammary Fat Pad Transplantation, Gene Function Analysis, Lentiviral Gene Modification, Stat5, Stat3, Estrogen Receptor, Breast Cancer, Review

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