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Cold Spring Harb Perspect Biol doi: 10.1101/cshperspect.a003178 published online August 25, 2010
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Hormone Action in the Mammary Gland

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A woman's breast cancer risk is affected by her reproductive history. The hormonal milieu also influences the course of the disease. The female reproductive hormones, estrogens, progesterone, and prolactin, have a major impact on breast cancer and control postnatal mammary gland development. Analysis of hormone receptor mutant mouse strains combined with tissue recombination techniques and proteomics revealed that sequential activation of hormone signaling in the mammary epithelium is required for progression of morphogenesis. Hormones impinge on a subset of luminal mammary epithelial cells (MECs) that express hormone receptors and act as sensor cells translating and amplifying systemic signals into local stimuli. Proliferation is induced by paracrine mechanisms mediated by distinct factors at different stages. Tissue and stage specificity of hormonal signaling is achieved at the molecular level by different chromatin contexts and differential recruitment of coactivators and corepressors.

Breast cancer is the most frequent cancer in women and the second leading cause of cancer deaths among women. To better understand the genetic alterations responsible for breast cancer, it is critical to first understand the mechanisms regulating normal mammary gland development. Increased interest in the field has led to the identification of a large number of genes important for mammary gland development (reviewed in Tanos and Brisken 2008).

A woman's risk for breast cancer is linked to her reproductive history and with her lifetime hormonal exposure; hormones also influence the course of the disease. The same hormones that affect breast carcinogenesis control postnatal mammary gland development. The mouse mammary gland has been instrumental in providing new insights into the mechanisms by which hormones act in the mammary gland.

A number of features make the mouse mammary gland a particularly attractive experimental system. Being the only organ that undergoes most of its development postnatally, it is particularly suited for studying developmental processes; it is readily amenable to experimental manipulation and can be easily accessed as it localizes to the underside of the ventral skin. Furthermore, mammary gland tissue is abundant; there are 5 pairs of mammary glands in mice, and cells can be isolated in large numbers. The versatile tools of mouse genetics can be combined with powerful tissue recombination

Editors: Mina J. Bissell, Kornelia Polyak, and Jeffrey Rosen
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techniques to generate chimeric glands, as we will illustrate in this article.

DEVELOPMENT OF THE MOUSE MAMMARY GLAND

Two major phases can be distinguished in mammary gland development: hormone-independent up to puberty, and hormone-dependent thereafter.

Hormone-Independent Mammary Gland Development

The mammary gland develops from a thickening in the ventral skin during embryogenesis (see Wysolmerski in this issue) that grows into a rudimentary ductal tree by birth. Until puberty, the mammary gland grows isometrically to the rest of the body. Although hormone receptors are expressed before puberty (Stumpf et al. 1980; Hovey et al. 2001; Grimm et al. 2002) and the fetus is exposed to high levels of maternal and placental hormones, it is generally held that the female mammary gland develops up to puberty in a hormone-independent fashion because no overt mammary gland phenotype is observed before puberty in a variety of hormone receptor deficient animals (see the following discussion). Elegant work with androgen receptor mutant mice revealed that males of certain strains do not have nipples, because during embryogenesis, testosterone secreted by the maturing testes induces apoptosis of the epithelial bud by activation of androgen receptor signaling in the mammary stroma (Durnberger and Kratochwil 1980). Perinatal exposure to exogenous hormones or endocrine disruptors, i.e., substances that can activate and/or inhibit hormonal signaling, can result in subsequent aberrant development (Bern et al. 1983, 1987), suggesting that even when hormone receptors are not physiologically required, their untimely activation can perturb development.

Hormone-Dependent Mammary Gland Development

Hormone-dependent mammary gland development occurs after puberty and results in ductal elongation; recurrent estrous cycles in adulthood trigger side branching; pregnancy enhances side branching and induces alveologenesis with lactational differentiation followed by involution at weaning (Brisken 2002). In the late fifties, a series of experiments defined the minimal hormonal requirements for mammary gland development in mice (Nandi 1958) and rats (Lyons 1958). Endocrine ablation was achieved by surgically removing the major sources of reproductive hormones from mature females, the ovaries, which secrete estrogens and progesterone, the pituitary gland, a major source of growth hormone (GH) and prolactin (Prl), and for some experiments the adrenal glands, which release cortisol and precursors of sex steroids (see Fig. 1). Hormone replacement in hormone-deprived animals established that additive and sequential treatment with 17-β-estradiol, progesterone, and prolactin in conjunction with cortisol and GH can recapitulate mammary gland development.

MECHANISMS OF IN VIVO HORMONE ACTION

Systemic versus Local Effects

Hormones act on multiple organs and affect each other's synthesis and secretion. Estrogens, for instance, control the reproductive tract and the gonads as well as the skeletal system and the cardiovascular system (Stampfer et al. 1991; McDonnell and Norris 1997; Couse and Korach 1999). They also act on the pituitary gland to stimulate prolactin synthesis and secretion (Fig. 1) (Scully et al. 1997). Prolactin controls the luteal body and hence progesterone synthesis in mice (Bachelot and Binart 2007), and induces transcription of ERα in different tissues (Frasor and Gibori 2003). Because of such interactions it is impossible to discern in physiological settings to what extent the effects of a given hormonal stimulus on the mammary gland are a result of direct hormone action on this tissue or secondary to stimulation of other organs. Through gene targeting in the mouse germ line, mice were generated that are unresponsive to individual reproductive hormones, because they lack the cognate receptors. All

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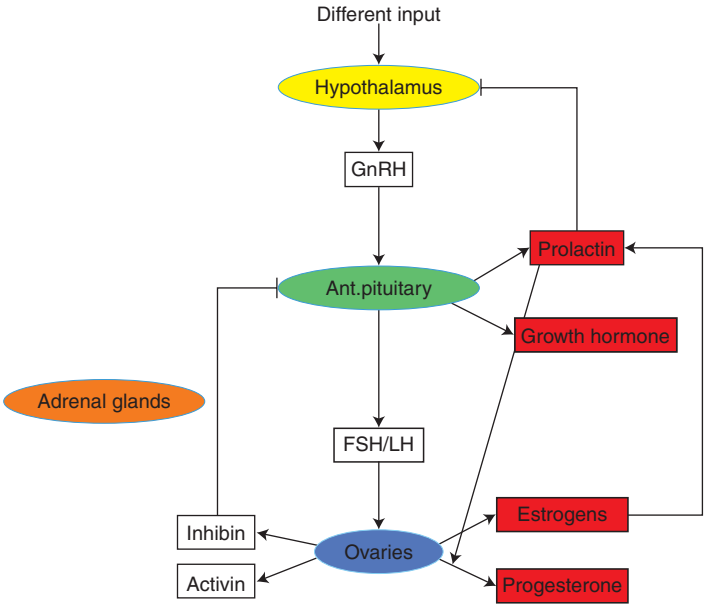

Figure 1. Scheme of female endocrine system. Different endocrine glands secreting mammotropic hormones are shown in ovals, hormones in boxes, highlighted in red are mammotropic hormones.

the receptor-deficient mouse strains are viable but have reproductive abnormalities; ERα, ERβ, PR, and PrlR deficient are sterile for different reasons (Lydon et al. 1995; Ormandy et al. 1997; Dupont et al. 2000; Antal et al. 2008), whereas GHR-/- mice have delayed sexual maturation (Zhou et al. 1997).

Estrogens

In ERα-/- females, mammary gland development is indistinguishable from that of wild-type (wt) littermates until the age of puberty, thereafter no development occurs as assessed by whole mount stereomicroscopy and histology. For the former, mammary glands are cleared of fat, stained, and visualized at 5- to 120-fold magnification. The entire gland, about 1−3 cm^3 in size can be examined up to a resolution that corresponds to several cell diameters. Histological analyses complement morphology at cellular resolution.

The ERα is expressed both in the mammary epithelium and the mammary stroma (Daniel et al. 1987). To assess the role of epithelial intrinsic ERα signaling in the context of wt stroma in a wt host, inguinal mammary fat pads of 3-week-old wt females were cleared of endogenous epithelium and engrafted on one side with ERα-/- epithelium and contralaterally with wt epithelium (Mallepell et al. 2006). When wt epithelium is grafted into such "cleared fat pads," it grows to fill the entire fat pad and behaves like endogenous epithelium (DeOme et al. 1959). Within a few weeks, the graft grows to fill the fat pad through dichotomous branching; cell proliferation concentrates at the tip of the ducts that enlarge to spoonlike shapes called terminal end buds (TEBs) (Daniel and Silberstein 1987). In contrast, ERα-/- mammary epithelium grafted contralaterally and hence exposed to the same hormonal milieu, fails to grow at all. During pregnancy, the wt grafts display side branching and alveoli bud off all over the ductal tree whereas the ERα mutant epithelium remains a rudiment (Mallepell et al. 2006). This indicates that epithelial ERα signaling is required for ductal elongation and, directly or indirectly, for subsequent side branching and alveologenesis.

To assess the role of stromal ERα signaling, ERα-/- fat pads were grafted onto the abdominal muscle wall of wt hosts, and a piece of wt

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epithelium was inserted into them, thereby generating mice that specifically lack ERα in the fat pad of one of their mammary glands. In these chimeric glands, grafted wt epithelium grows out normally and shows alveolar differentiation whereas endogenous mutant epithelium coexisting in the same fat pad remains rudimentary (Mallepell et al. 2006). Thus, the presence of the stromal ERα is not required for mammary gland development.

ERβ-/- females show some delay in side branching, which may be attributable to irregular estrous cycles related to perturbed ovarian function in these mutants that results in decreased progesterone secretion (Antal et al. 2008). Hence the physiological role of ERβ in the mammary epithelium and the mammary stroma, where it has been reported to be more highly and widely expressed than the ERα (Cheng et al. 2004), remains unclear. GPR30, a G-protein coupled receptor, has been implicated in mediating rapid nongenomic estrogen signaling in different cellular systems, including breast cancer cell lines (reviewed in Prossnitz et al. 2007); when deleted from the mouse germ line, no abnormalities in the reproductive system were found (Otto et al. 2009).

Progesterone

Like ERα, PR is expressed in both epithelial and stromal compartments in the mouse mammary gland (Haslam and Shyamala 1981; Haslam 1989). PR-/- epithelial grafts grow out normally when grafted to cleared fat pads of wt hosts but fail to side branch and do not form alveoli, indicating that epithelial intrinsic progesterone receptor signaling is required for side branching and alveologenesis. Deletion of PR in the stroma did not affect mammary gland development as assessed by whole mount microscopy (Lydon et al. 1995; Brisken et al. 1998). PRs are composed of two proteins that are expressed from a single gene as a result of transcription from two alternative promoters (Kastner et al. 1990) both of which are expressed in the mouse mammary gland (Aupperlee et al. 2005). Characterization of the mutant strains lacking one or the other form revealed that PR-B is uniquely required for mammary gland development (Mulac-Jericevic et al. 2000, 2003).

Prolactin and Others

Grafting experiments with PrlR-/- epithelium showed that ductal outgrowth and side branching can occur in the absence of epithelial PrlR signaling (Brisken et al. 1999). However, the Prl signaling pathway is required for alveologenesis and differentiation of MECs into milk producing cells during late pregnancy. Morphological hallmarks of secretory differentiation such as fat droplets and "granular" cytoplasm are absent. Expression of specific differentiation markers such as the milk proteins β-casein and whey acidic proteins (WAP) mRNA is lost in PrlR-/- epithelium and STAT5a phosphorylation is undetectable (Brisken et al. 1999; Gallego et al. 2001). Wt epithelium grafted into PrlR-/- stroma developed normally (Ormandy et al. 2003).

GHR-/- mammary epithelium develops and differentiates normally when grafted to cleared fat pads indicating that epithelial GHR signaling is not limiting for mammary gland development (Gallego et al. 2001). It was suggested that GH acts on the mammary stroma because injections of GH resulted in STAT5a phosphorylation and STAT5a/b heterodimer formation to a comparable extent in intact mammary glands and cleared fat pads; however, it cannot be excluded that effects of the hormone on other organs may be involved as well. GH induces the production of IGFs in the liver, and IGF signaling is important for mammary gland development. Similarly, GH injection was shown to elicit Stat5 phosphorylation in myoepithelial cells as detected by immunohistochemistry (LeBaron et al. 2007). For a more extensive review of GH and Prl lactogenic functions see (Trott et al. 2008).

Taken together, a picture emerges in which estrogens, progesterone, and prolactin act sequentially on the mammary epithelium in synergy with corticosteroids to orchestrate mammary gland development in the presence of GH acting possibly via stromal and epithelial GHRs (Fig. 2). During puberty, estrogen levels increase first to set the stage for progesterone,

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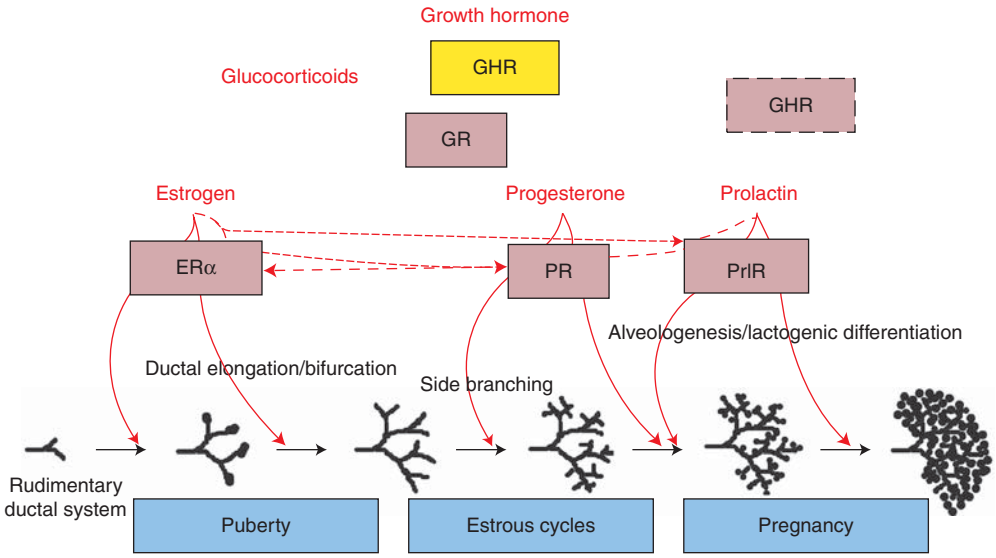

Figure 2. Control of mammary gland development by hormones. Different stages of mammary gland development are depicted. All hormone receptors are required in the mammary epithelium (pink boxes) with the exception of the GHR that is required in the stroma (yellow box) but also signals in the epithelium (dotted pink box). Red arrows indicate when different hormones are limiting with growth hormone and glucocorticoids being required throughout mammary gland development. Dotted arrows illustrate hormonal regulation of HR expression.

by inducing the expression of the PR (Haslam and Shyamala 1979). This so-called "estrogen priming" occurs in most progesterone target tissues. Cyclic secretion of progesterone is established as the mouse attains sexual maturity and this coincides with the ducts reaching the edges of the fat pad by dichotomous branching. Once side branches are established by midpregnancy, further alveologenesis requires epithelial prolactin signaling (Brisken 2002). This sequential action ensures that the distinct morphological steps occur in an orderly manner; in this way, all of the ducts are established before alveoli bud and they find adequate space to unfold and to be drained efficiently.

This sequential alignment defined by the stages at which particular hormone receptors are limiting should not detract from the fact that at any given time all hormones are present albeit at different concentrations in the blood and/ or locally and that they interact with each other at multiple levels. As examples, prolactin is only limiting for alveologenesis, yet, it may synergize with estrogens and progesterone at different levels during the preceding stages of ductal outgrowth and side branching. Prolactin and progesterone may enhance ductal outgrowth by inducing ERα expression. Furthermore, targeted gene deletion in the germ line may mask physiologic functions of a given gene product as mutant tissues may have time during development to compensate for the loss of a particular gene product. Plasticity may result in compensation, and it is conceivable that if a hormone receptor was efficiently abrogated at a later stage, different and/or more severe phenotypes would be discerned.

The endocrine glands determine the hormonal milieu and are strictly controlled by the hypothalamo-pituitary axis, which in turn reacts to feed back from the periphery (see Fig. 1). In addition to the main players, which are limiting, a number of additional systemic factors are likely to be involved in fine-tuning the system such as vitamin D (Zinser et al. 2002; Zinser and Welsh 2004) and thyroid hormones. In recent years it has become apparent

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that adipose tissue, in particular visceral, is actively secreting adipokines and cytokines such as leptin, adiponectin, and TNFα, which have important implications for metabolic syndromes (Antuna-Puente et al. 2008). Evidence has accumulated that some of these factors may affect breast carcinogenesis by altering the tumor microenvironment; in the mouse, adiponectin haploinsufficiency promotes MMTV-PyVT induced mammary tumors in different genetic backgrounds (Lam et al. 2009). The physiologic role of the mammary fat pad as an endocrine gland, the role of the factors it secretes, and the mechanisms through which they affect mammary gland development have not been explored. Intriguingly, mice lacking leptin (Lep^{ob}/Lep^{ob}) or the leptin receptor ($Lepr^{db}/Lepr^{db}$) have a rudimentary, atrophic ductal tree at 1 or 2 yr of age (Hu et al. 2002). The mammary epithelium itself secretes PTHrP, important for nipple development (Kobayashi et al. 2005) and prolactin that enhances mammary epithelial cell proliferation during lactation (Naylor et al. 2003). Finally, data from humans indicate that local concentrations of 17-β-estradiol and its metabolites reflect not only serum estrogen levels but also local production resulting from conversion of C19 steroids such as androstendione and testosterone into estrogens by aromatase expressed within the gland (Santen et al. 2009 and references therein) highlighting that many other factors impinge on hormone signaling.

Intercellular Signaling (Paracrine Mechanisms)

How does activation of hormone receptors by their respective ligands elicit proliferation and morphogenesis? A closer look at the mammary epithelium reveals that it consists of two cell compartments, luminal and basal. Luminal cells are connected by tight junctions and form a single layer of polarized epithelium around the ductal lumen. The basal compartment comprises all the cells that do not touch the lumen; these include progenitors and myoepithelial cells, which are contractile, form a meshwork around the luminal cells, and play a role in milk ejection during lactation. About a third of the luminal epithelial cells express ERα and PR (Fig. 3), and expression largely overlaps, at least in human breast epithelial cells (Clarke et al. 1997).

Across species, most proliferation occurs in steroid receptor negative cells in the adult mammary epithelium with only a few ERα positive cells incorporating labeled nucleotides (Clarke et al. 1997; Russo et al. 1999; Seagroves et al. 2000; Grimm et al. 2002). Because the ERα protein is rapidly degraded by the proteasome upon transactivation (Reid et al. 2003), it was proposed that ERα expression is down modulated in cells that have entered the cell cycle as a consequence of estrogen stimulation (Cheng et al. 2004). When ERα-/- MECs were mixed with wt MECs in vitro and this mixture was subsequently used to reconstitute cleared fat pads, it was demonstrated that in the presence of surrounding wt MECs, ERα-/- MECs proliferate extensively and contribute to all cellular compartments in the mammary gland, that is, body and cap cells of TEBs and myoepithelial and luminal cells of the mature ducts. These data indicate that estrogens can elicit proliferation by a paracrine mechanism and that ERα-/- cells can actively proliferate in response to estrogens (Mallepell et al. 2006). The same is the case for progesterone, as PR-/- MECs form side branches and alveoli in the context of chimeric epithelia with wt MECs (Brisken et al. 1998). Depending on the developmental stage and the predominant hormonal stimulus, the scenarios at the cellular level are different.

During puberty, estrogens are the major mitogenic stimulus and signal via ERα. In sexually mature females, estrogens elicit little proliferation but are permissive for the strongly mitogenic effects of progesterone. Experiments with amphiregulin-/- mammary epithelia grafted into cleared wt fat pads, revealed that amphiregulin is an essential mediator of estrogen-induced proliferation during puberty. Amphiregulin is the only EGF family member whose transcription is strongly induced by 17-β-estradiol in the peripubertal mammary gland (Ciarloni et al. 2007). Amphiregulin is a membrane-anchored protein that can be

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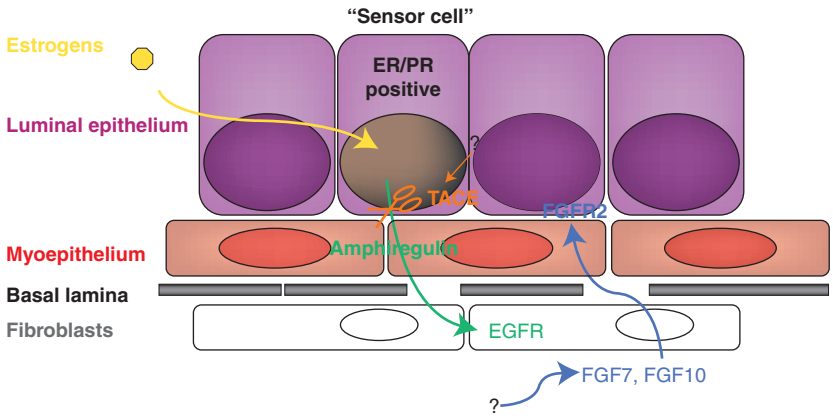

Figure 3. Local control of mammary gland development by estrogens. For explanations see text. As cell proliferation in response to estrogens increases at ductal tips the basal lamina becomes discontinuous until it eventually disappears right around the cap cells of the growing TEBs.

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cleaved and released by ADAM17/TACE (Sunnarborg et al. 2002). Consistent with amphiregulin being released and thereby activated by TACE, in vivo ablation of ADAM17 from the epithelium blocks ductal outgrowth mimicking both amphiregulin and ERα-/- phenotypes (Sternlicht et al. 2005). Amphiregulin binds to and activates the epidermal growth factor receptor (EGFR), which is expressed in the stromal compartment during ductal elongation (Schroeder and Lee 1998). Recombination experiments with EGFR-/- tissue showed that stromal EGFR expression is required for ductal outgrowth whereas epithelial deletion does not interfere with ductal development (Wiesen et al. 1999). Initially, this epithelial-stromal crosstalk may not be efficient as the amphiregulin secreted by ERα positive luminal cells needs to be transported through the myoepithelial layer and the basal lamina before it can dock to its cognate receptor on a stromal fibroblast. However, as hormonal stimulation persists, proliferation increases substantially at the tips of the ducts and they enlarge to form spoon-shaped structures called terminal end buds (TEBs). The bulk of cells within these structures are called body cells. The outer cell layer of the TEBs is continuous with the myoepithelium of the subtending mature duct and consists of cap cells, which lack intercellular adhesion. Around the TEB the basal lamina is disrupted and the outer epithelial cells, the highly proliferative cap cells are in direct contact with stromal cells.

The question arises as to the identity of the mitogenic signal that makes MECs proliferate. Very attractive candidates are FGF7 and 10. Messenger RNA expression of both factors is induced in the mammary fat pad following estrogen stimulation, and it is conceivable that induction of their mRNAs is mediated by the EGFR in the stroma in response to amphiregulin. Their cognate receptor, FGFR2, is required in the epithelial compartment for ductal elongation (Lu et al. 2008). When FGFR2 was deleted at later stages through recurrent activation of the MMTV-cre in response to progesterone stimulation during side branching, the mutant MECs persisted, indicating that FGFR2 is specifically required for estrogen-induced proliferation.

In case of progesterone and prolactin, the local signaling circuitry is less well understood. Wnt-4 is required in the mammary epithelium for side branching (Brisken et al. 2002) and the TNF family member RANKL was identified as a progesterone target gene. Based on the observation that RANKL protein localizes to PR positive cells, which occur next to BrdU incorporating cells, it was proposed that RANKL is a paracrine mediator of progesterone-induced

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proliferation (Mulac-Jericevic et al. 2003). Consistent with this scenario, functional data show that ectopic expression of RANKL using a MMTV transgene results in side branching in the absence of pregnancy (Fernandez-Valdivia et al. 2009).

IGF-2 has been implicated as an important mediator of PrlR signaling upstream of cyclin D1 mediating prolactin effects (Brisken et al. 2002; Hovey et al. 2003). TGFβ signaling has been implicated as a growth inhibitory signal in mammary gland development acting through Wnt5a and important in both estrogen and progesterone induced morphogenesis (Roarty and Serra 2007 and references therein). An overview of the factors that have been implicated in mammary gland development is shown in Figure 4.

The paracrine mode of action ensures that the behavior of different cells is coordinated; this is important as epithelial cell proliferation in a glandular organ such as the breast results in morphogenesis with new ducts and alveoli being formed. Furthermore, in this way the hormonal signals in nano or picomolar ranges are amplified, a scenario that is reiterated clearly at the molecular level by the use of transcriptional coactivators as discussed later.

Molecular Mechanisms of Differential Hormone Action

How can the steroid hormones elicit completely different effects in different tissues and developmental contexts? Identification of new players in nuclear receptor (NR) signaling has shed new light on the molecular underpinnings in recent years.

Structural Features of Estrogen and Progesterone Receptors

The chemical signals of estrogen and progesterone are transduced by their specific intracellular steroid receptors (SRs). SRs are functionally composed of three main domains: a hormone-independent activation function 1 (AF1 domain), a DNA-binding function (DBD domain), and hormone-dependent activation function 2 (AF2 domain) (Fig. 5) (Mangelsdorf et al. 1995). The estrogen receptor α (ERα) gene, which produces a protein of 595 amino acid residues with a molecular mass of 66 kDa, is composed of six structural subdomains corresponding to the three functional regions described above (Evans 1988; Bourguet et al. 2000; Klinge 2000; Nagy and Schwabe 2004).

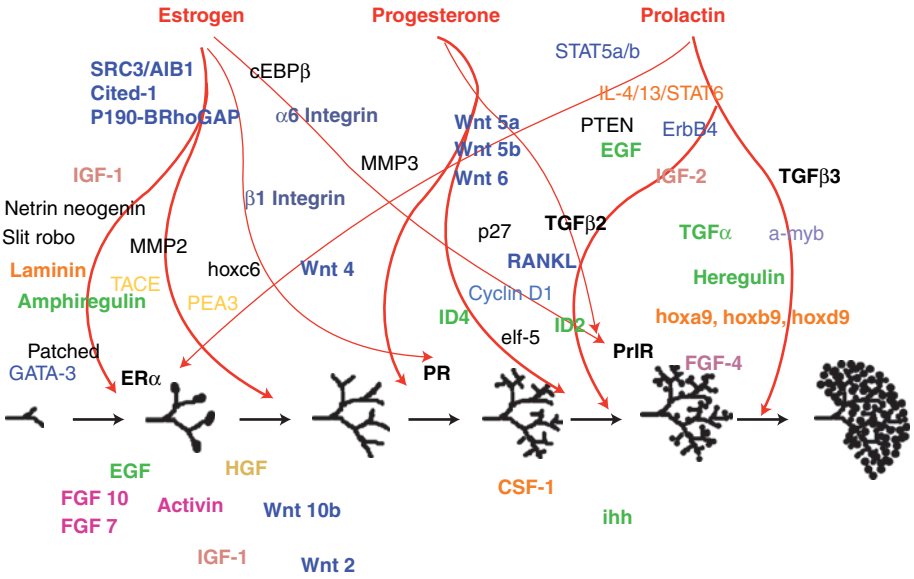

Figure 4. Factors involved in mammary gland development. Work of many laboratories led to the identification of many genes important in mammary gland development that are summarized in the scheme.

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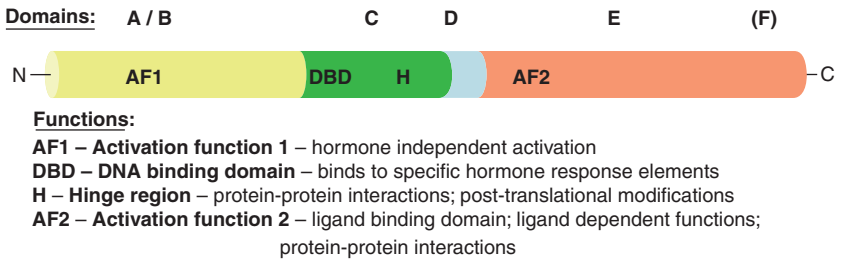

Figure 5. General structure of nuclear hormone receptors. Steroid receptors differ in details, but are generally composed of multiple (5–6) structural domains (A–F), and functional domains (in colors). The functions of these receptor regions are listed in the figure: AF1 (activation function one); DBD (DNA binding domain); H (hinge domain); AF2 (activation function two). The AF1 and AF2 provide the main surfaces that interact with other transcription factors to transduce the signal of the hormonal ligand, which binds to the ligand binding domain (LBD, orange color).

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The A and B structural subdomains of ERα functionally belong to the AF1 region of the receptor. This region is implicated in the hormone-independent transcriptional activation of the receptor. The C subdomain represents the DNA binding domain (DBD) of the molecule. It is composed of two zinc-finger motifs responsible for receptor interaction with the DNA. The D subdomain of ERα, or the hinge region is a 39-amino-acid long linker between the DNA and ligand binding regions (LBD) of ERα. Functionally, it contains a nuclear localization signal (NLS) and it is implicated in interaction with some coregulator molecules. The E structural subdomains of the ERα molecule encompass the ligand-binding surface and the AF2 functional domain. The AF2 domain is responsible for the ligand dependent transcriptional activity of ERα. The ligand-binding region is composed of 5 α helixes (helixes 3, 6, 8, 11, and 12), which form a hydrophobic ligand-binding cleft (Evans 1988; Bourguet et al. 2000; Klinge 2000; Nagy and Schwabe 2004; Pike 2006). Upon binding to E2, this region undergoes a conformational change such that helix 12 is displaced so as to cover the ligand-binding pocket. This change in the position of helix 12 creates a new interacting plane at the surface of the molecule, which is then used for the recruitment of coactivator molecules. Coactivator proteins contain one or more LXXLL helical amino acid motifs that interact with liganded ERα (Klinge 2000; Lonard and O'Malley 2007, 2008a, 2008b). Finally, there is the carboxy-terminal F subdomain whose role in the receptor is less clear, but has been shown to be involved in part in receptor dimerization (McKenna et al. 1999).

With minor variations, the functional domains contained in the ERα and ERβ molecules also can be found in the PR, and all other steroid hormone receptors. The PR gene is unique in that it codes for two receptor isoforms (PR-A and PR-B), which display overlapping but also distinct gene regulatory properties in relaying the progesterone signal. Distinct tissue-specific reproductive responses to progesterone exhibited by these two progesterone receptor isoforms are caused by regulation of distinct subsets of progesterone-dependent target genes by the individual PR isoforms. (Conneely et al. 2003).

Mechanisms of Regulation of Steroid Receptor Actions by Coregulators

In steroid receptor containing cells, a new class of molecules, termed coregulators, was discovered over a decade ago (Oñate et al. 1995). The coregulators are generally enzymes capable of modifying chromatin proteins, the basal transcriptional machinery, and other coregulators. The coregulators are composed of coactivators, which provide positive enhancement to gene expression, and corepressors, which are

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employed to suppress gene expression. These regulatory molecules provide the ability to fine-tune our genes and activate them in functional combinations. Recently, we have come to realize that coactivators are the likely "master regulators" of our genome, capable of coordinately activating subgroups of genes that are required for specific physiologic processes such as reproduction, growth, inflammation, or metabolism (O'Malley 2007). DNA-binding transcription factors, such as NRs, bind nearby to genes and mark them for activation or repression, functions subsequently affected by the recruitment of the coregulators. The coregulators exist and function in large multiprotein complexes that are recruited by NRs to target genes in an ordered sequence to provide the many enzyme capacities required for modifying histones and other coactivational proteins for transcription (Fig. 6). Subreactions of transcription mediated by coactivator complexes include chromatin modification and remodeling, initiation of transcription, elongation of RNA chains, mRNA splicing, and even, degradation of the activated NR-coregulator complexes and termination of the transcriptional response (Lonard and O'Malley 2007). Surprisingly, recent reports show that coactivators even can influence cellular reactions outside the nucleus such as mRNA translation, mitochondrial function, and cell motility.

When considering the plethora of functions that NRs play in tissues, it is not surprising that a relatively large number of coregulators appear to be involved in human breast cancer. The cellular concentrations of coactivators and corepressors are critical to their functional potential. A high concentration of a coactivator will lead to an amplified signal within a downstream transcription factor action pathway, as well as a much faster response to environmental signals. However, available data suggest that the key to understanding the true diversity of coregulator functions lies in first understanding the surprisingly extensive "posttranslational coding" that has been discovered to exist in the coregulator proteins (Han et al. 2009). Such posttranslational modifications (PTMs) include but are not limited to phosphorylation, methylation, ubiquitination, SUMOylation and acetylation. In the case of coregulators, it has been observed that a combination of such modifications can lead to multiple functionally distinct activities for the same primary sequence protein. A "differentially coded" coactivator is now predisposed to form different multimeric coactivator complexes, and, thereby, is directed to interact with distinct genes and to perform different compartmental functions in a cell. For example, over 40 separate modifications of the SRC-3 coactivator have been determined to date. In total, this plethora of PTMs creates an enormous combinatorial "potential" of $(2)^{40}$ for SRC-3. This vast PTM "potential complexity" likely is never fully used in cells of an animal. Nevertheless, it can be speculated that every single PTM on a protein results in a modified molecule with "some" altered function. Thus, it is an inescapable conclusion that multiple combinations of PTMs can bestow a huge array of distinct and diverse functions to any one coregulator protein.

Together with SRC-1 and SRC-2 (GRIP1/TIF2), SRC-3 (AIB1/ACTR/RAC3/pCIP/TRAM1) completes the structurally related p160 family of coactivator proteins, whose carboxy-terminal domains mediate interactions with histone acetyltransferases and the coregulators CBP and p300, whereas the amino-terminal basic helix-loop-helix/PAS-containing domains interact with additional co-coregulators such as CoCoA (coiled-coil coactivator, GAC63 (Grip1-associated coactivator 63 and CARM1 (Coactivator-associated arginine methyltransferase 1. SRC-3 activation and interactions with co-coregulators are initiated by posttranslational phosphorylations, whereby different combinations of site-specific phosphorylations provide interaction specificity for different co-coregulators and transcription factors.

Regulation of Coregulator Concentration and Activity

Ubiquitinylation is another posttranslational modification that is essential for the regulation of SRC-3 by cellular signaling. It was recently demonstrated that transcriptional activation

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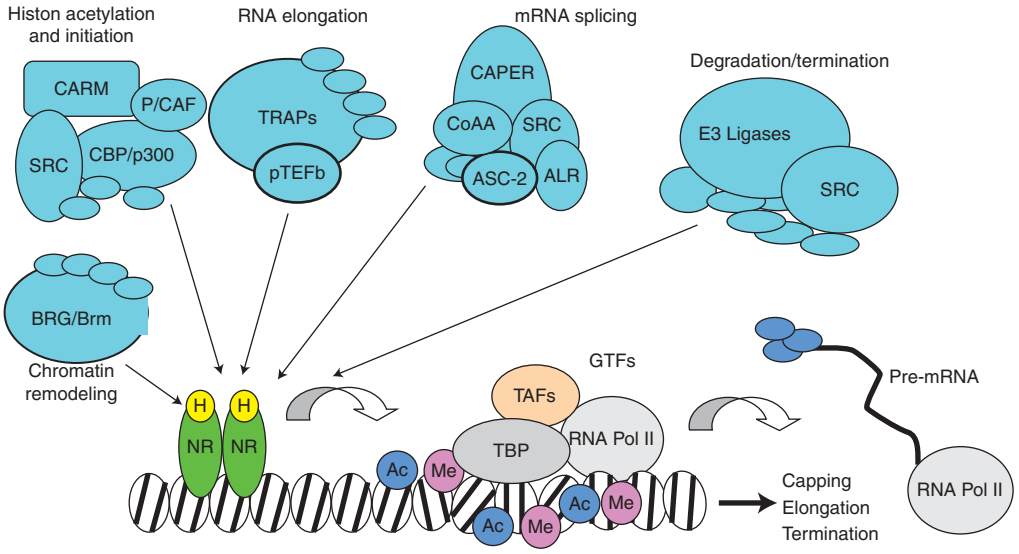

Figure 6. Nuclear Receptor (NR) dependent transcription, RNA splicing, and termination. NR regulated transcription begins by translocation of a hormone activated nuclear receptor dimer to hormone binding sequences in DNA near target genes. The receptor then must recruit, in sequence, a series of protein complexes that carry out all of the subreactions of DNA transcription: BRG/Brm complex regulates chromatin (nucleosome) remodeling; SRC/CARM/pCAF/CBP covalently modifies nucleosomes through mainly acetylation; TRAPs/pTEFb allows elongation of RNA polymerase on the gene; CAPER/CoAA/ASC-2/SRC/ALR provides splicing regulation; and E3 ligases bound to SRCs lead to degradations of the activated receptor and also the coregulators at the site of gene expression (after a short period of function). The General Transcription Factors (GTFs; TBP/TAFs) allow RNA polymerase to functionally transcribe the gene. Capping, elongation, termination are general aspects of RNA synthesis that result in the production of pre-mRNAs.

and turnover of SRC-3 are events controlled by phosphorylation-dependent mono-ubiquitinylation, which first super-activates the molecule for specific gene transcriptional enhancement. Ultimately, however, the transition from mono-ubiquitinylation to long-chain polyubiquitinylation leads to SRC-3 degradation. Because the course of polyubiquitinylation is processive during the transcriptional activation of transcription factors, this "phosphorylation-dependent ubiquitinylation" functions as a "transcriptional time clock" to first provide activation, and then to ultimately limit the lifetime of the PTM-activated coactivator (Lonard and O'Malley 2008b). SRC-3 also is regulated by a posttranslational modification that influences its structural association dynamics with other co-coactivators. Peptidyl-prolyl isomerase 1 (Pin1) catalyzes the cis/trans isomerization of proline residues adjacent to phosphorylated serine/threonine-P bonds to induce conformational changes in the SRC-3 protein and enhance the interactions between SRC-3 and other coactivators such as CBP/p300. In contrast, CARM1 methylates SRC-3 and dissociates it from its active coactivator complex.

Importantly, a recent study demonstrated that a previously undiscovered isoform of SRC-3 that lacks the amino-terminal nuclear localization signal is produced from the SRC-3 (NCoA3) gene. This cytoplasmic isoform, is phosphorylated and activated by the PAK-1 (p21 activated kinase) oncogenic kinase. When activated, this isoform, which lacks a nuclear translocation signal, functions at the cell membrane by interdigitating between the EGF receptor and FAK (focal adhesion kinase, the main motility kinase of cells). In this way, it acts as an adaptor to allow transduction of the signal for motility and invasion from the

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EGFR (and Her2) receptor to the FAK enzyme. It is an impressive example of a nuclear coactivator gene integrating nuclear growth gene responses (by full-length SRC-3) with a membrane growth signal (by the shorter isoform) for an important cellular response. This collaboration has great pathologic impact in that the combination of SRC-3 gene overexpression and EGFR (Her2) overexpression is a deadly combination in human breast cancer, leading to early tamoxifen resistance and rapid progression of the disease (Long et al. 2010).

The oncogenic potential for coactivators is now generally accepted. Coactivators designed as "master regulators" for cell growth coordinately regulate the expressions of many genes that must be in play simultaneously for effective growth. SRC-3/AIB1 is over expressed in 40%–65% of human breast cancers and is amplified in up to 10% of breast cancers; its cellular and in vivo oncogenic activities have been demonstrated in multiple labs. Tumorigenic activity of SRC-3 is substantiated by studies in transgenic mice that overexpress SRC-3 and develop spontaneous malignant mammary tumors (Xu et al. 2009). In contrast, SRC-3 knockout mice are resistant to chemical carcinogen-induced and viral-induced mammary tumorigenesis. SRC-$3^{-/-}$ mice also show resistance to induced prostate cancer progression. All of these results are consistent with the idea that SRC-3 is a potentially powerful oncogene. In the lymphatic system, however, SRC-3 can act paradoxically as a tumor suppressor because B-cell lymphomas develop in gene-deleted mice. These two faces of SRC-3 highlight the fact that SRC-3 is a versatile protein, allowing the cell to decide between proliferation or growth suppression in a cell and signal context-dependent manner. Although the majority of studies have been directed toward SRC-3, SRC-1 and SRC-2 are not without relevance to cancer. SRC-1 has been shown to be necessary for tumor metastasis in mice and has been suggested as a reliable marker for disease reoccurrence in human breast cancer. The combination of overexpression of SRC-3 and Her2 is deadly, producing early tamoxifen resistance and rapid progression to death for patients (Osborne et al. 2003). SRC-3, SRC-1, and other coactivator proteins/genes are now being used as markers for breast cancer prognosis and estimates of recurrence of disease after treatment (Redmond et al. 2009).

CONCLUDING REMARKS

Powerful FACS sorting approaches and a growing repertoire of markers for different cell populations within the mammary gland now allow the research community to explore the hitherto hidden organization of cells within different compartments of the mammary gland. Ever more powerful imaging techniques are used to unravel interactions with immune cells when not so long ago these were a black box. Major challenge remain in the manipulation of selective stromal components with first steps made with new Cre lines (Trimboli et al. 2008), and in understanding how the findings in the mouse model can be extrapolated to human beings. Our appreciation of how mammary gland and breast cancer development are orchestrated by systemic hormones promises to continue to evolve quickly.

ACKNOWLEDGMENTS

The authors thank F. Koerner for critical reading of the manuscript.

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