Mammary Gland Growth Factors: Roles in Normal Development and in Cancer

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Normal development of the mammary gland proceeds via interactions between the epithelium and the mesenchyme that start during embryogenesis and continue during pubertal outgrowth and differentiation. The function of specific peptide growth factors that bind members of the receptor tyrosine kinase family and the cytokine receptor family are required at each stage. In many cases the peptides are produced in one compartment and act on receptors in the other compartment. One of the striking differences between normal development and cancer is the loss of this cross-talk. Mammary tumor cells often produce a peptide and express the receptor on the same cell leading to autocrine activation of signaling pathways, a mechanism that is characteristic for cancer cells. We will discuss different peptides in the context of normal development and cancer in this review.

Development of the mouse mammary gland begins at embryonic day 10.5 with formation of the milk line, a thickening of the ectoderm that extends from the anterior to the posterior limb buds. By E11.5 five placode pairs invaginate into the surrounding stroma and undergo limited branching morphogenesis. At birth a rudimentary ductal tree is present in a stromal fat pad. At the onset of puberty ovarian hormones induce ductal elongation until the fat pad is filled with a system of ducts and side branches. During pregnancy the glands fill with secretory alveolar units that on parturition produce copious amounts of milk for the offspring. As the pups are weaned, these alveoli are removed by programmed cell death coupled with extensive tissue remodeling, an event

termed involution. All these processes are under the control of a wide variety of hormonal and peptide factors. In this review, which is concentrated on peptide growth factors, we will discuss those factors that have important roles in normal development and have been implicated in breast cancer.

RECEPTOR TYROSINE KINASES AND PEPTIDE LIGANDS IN NORMAL DEVELOPMENT AND CANCER

The superfamily of receptor tyrosine kinases (RTK) includes approximately 60 members divided into 19 subfamilies. Each receptor has an extracellular ligand binding region, a single membrane-spanning region, and a cytoplasmic

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tyrosine kinase-containing domain. In this review we will discuss the epidermal growth factor receptor (EGFR) or ErbB family and the EGF-related ligands, the fibroblast growth factor receptor (FGFR), and FGFs as well as insulin-like growth factors (IGFs) and their receptors, mentioning their involvement in normal development of the mammary gland and in breast cancer.

EGF FAMILY OF GROWTH FACTORS AND THEIR RECEPTORS

There are four members of the ErbB RTK family: EGFR, ErbB2, ErbB3, and ErbB4. Peptides in the EGF family bind ErbB receptors and based on their receptor specificity are divided into three groups: The first includes EGF, transforming growth factor (TGF)- α , amphiregulin (AR), and epigen (EPG), which bind EGFR; the second includes betacellulin (BTC), heparin-binding EGF (HB-EGF), and epiregulin (EPR), which bind both EGFR and ErbB4; the third group, the neuregulins (NRGs), forms two subgroups based on their capacity to bind ErbB3 and

ErbB4 (NRG-1 and NRG-2) or ErbB4 (NRG-3 and NRG-4) (Hynes and Lane 2005) (Fig. 1).

Ligand binding initiates signaling by causing specific homo- or heterodimeric receptor formation and activation of the cytoplasmic kinase domain that phosphorylates tyrosines in the tail region of each receptor. Phosphorylation triggers the association of specific signaling molecules whose binding initiates downstream signaling events. Of note, ErbB3 has impaired kinase activity and only acquires signaling potential, i.e., tyrosine phosphorylation, when dimerized with another receptor. Furthermore, ErbB2 does not bind any of the EGF-family ligands, however, it is activated via heterodimerization with the other ligand-bound receptors (Hynes and Lane 2005) and is their preferred heterodimerization partner (Graus-Porta et al. 1997).

EGF FAMILY OF PEPTIDE GROWTH FACTORS AND RECEPTORS

Members of the FGF ligand family and their receptors form a complex network with four

Figure 1. EGF-family peptides and ErbB Receptors. The four ErbB receptors are shown: (1) EGFR, (2) ErbB2, (3) ErbB3, and (4) ErbB4. The EGF-family peptides bind specific ErbB receptors: EGF, TGF- α , AR, and EPG bind EGFR; BTC, HB-EGF, EPR have dual specificity, binding both EGFR and ErbB4; NRG-1 and NRG-2 bind ErbB3 and ErbB4; NRG-3 and NRG-4 bind ErbB4 (Hynes and Lane 2005). Various roles for specific ligands (boxed in red) and all the ErbB receptors have been described in normal mammary gland development. In breast cancer, overexpressed ErbB2 together with ErbB3 function as a unit to drive proliferation of specific subsets of tumors (Holbro et al. 2003). EGFR is overexpressed in 30%–60% of basal-like breast cancers. See text for more information.

FGFR genes that undergo alternative splicing to yield minimally seven FGFRs. Alternative splicing of FGFR 1–3 in the D3 region (III) of the ectodomain yields, e.g., FGFR1-IIIb and FGFR1-IIIc, that bind different FGFs and are expressed in epithelial or mesenchymal compartments, respectively. The 18 FGFs are divided into six subfamilies; the FGF 11–14 subfamily has been renamed fibroblast homologous factors because their core structure is similar to other FGFs, however, several key residues for receptor binding are missing and they do not bind FGFRs (reviewed in Itoh and Ornitz 2004; Beenken and Mohammadi 2009).

Each FGF ligand binds a specific FGFR (Fig. 2), which enforces receptor dimerization, kinase activation and autophosphorylation of specific tyrosine residues in the cytoplasmic

domain (Mohammadi et al. 1996; Lew et al. 2009). In addition to the receptor, FRS2, a major adaptor linking FGFRs to the ERK and PI3K pathways becomes phosphorylated on specific tyrosine residues (Kouhara et al. 1997; Ong et al. 2001).

INSULIN, IGFs, AND THEIR RECEPTORS

The peptides insulin and IGF1 bind to various members of the insulin receptor (IR)-IGF1 receptor (IGF1R) family. The receptors are tetrameric structures composed of half receptors each of which has an extracellular ligand-binding α chain and a transmembrane spanning β chain with cytoplasmic tyrosine kinase activity. IGF1 binds IGF1R as well as a hybrid composed of an IR and an IGF1R. Moreover,

FGF subfamilies		FGFR activation						
		R1IIIb	R1IIIc	R2IIIb	R2IIIc	R3IIIb	R3IIIc	R4
FGF1								
	FGF1	+	+	+	+	+	+	+
	FGF2	+	+		+		+	+
FGF4								
	FGF4		+		+		+	+
	FGF5		+		+		(+)	
	FGF6		+		+			+
FGF7								
	FGF3	+		+				
	FGF7			+				
	FGF10	+		+				
	FGF22	+		+				
FGF8								
	FGF8		+		+		+	+
	FGF17		+		+		+	+
	FGF18				+		+	+
FGF9								
	FGF9		+		+	+	+	+
	FGF16		(+)		+	+	+	+
	FGF20		+		+	+	+	+
FGF19 (endocrine)								
	FGF19				+		+	+
	FGF21		+				+	+
	FGF23		+				+	+

Figure 2. Members of the FGF family are divided into six subgroups of closely related peptides. Each ligand binds to a specific subset of the FGFRs. () refers to the fact that there is still uncertainty regarding the binding. The table summarizes results from many laboratories and some relevant publications are as follows: (Ornitz et al. 1996; Zhang et al. 2006; Kurosu et al. 2006, 2007; Suzuki et al. 2008; Schwertfeger 2009).

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the bioavailability of IGF1 is controlled by IGF binding proteins (IGFBP) (reviewed in De Meyts 2004). As for other RTKs, ligand binding stimulates multiple intracellular pathways. One specific characteristic of the IR-IGF1R family is their dependence on insulin responsive substrate (IRS) family members, in particular IRS-1/2, for intracellular signaling.

ROLES FOR EGF-FAMILY PEPTIDES, FGFs, AND IGF1 AND THEIR RECEPTORS IN MAMMARY GLAND DEVELOPMENT

Embryonic Development of the Mammary Gland

Development of the mouse mammary gland begins in at embryonic day E10.5 and by E11.5, five placode pairs invaginate into the surrounding stroma and undergo branching morphogenesis. FGF10 and its receptor FGFR2-IIIb have important roles during embryonic mammary gland development. Starting at E10.5, somatic-expressed FGF10 acts on FGFR2-IIIb in the ectoderm to initiate induction and positioning of the mammary line and placode formation. The ligand and the receptor are both essential for induction of four of the five placode pairs (Mailleux et al. 2002; Veltmaat et al. 2006). Considering upstream regulators of the pathway, it has been shown that FGF10 levels are reduced in somites lacking the transcription factor GLI3, and the mammary line and placode three fail to form in its absence (Veltmaat et al. 2006).

NRG3 was discovered as a hypomorphic allele causing hypoplastic mammary buds in *ska* mutant mice (Howard et al. 2005). NRG3 is expressed in the mesenchyme adjacent to sites where placodes form and acts to promote mammary morphogenesis. ErbB4, the NRG3 receptor, as well as the other ErbB receptors, are expressed at sites of epithelial–mesenchymal interactions during morphogenesis of the embryonic mammary gland (Wansbury et al. 2008). Intriguingly, embryonic mammary placode formation appears to be normal in ErbB4 null mice (Tidcombe et al. 2003). Indeed, embryos deleted for each of the four ErbB

receptors appear to form normal mammary placodes (Wansbury et al. 2008). This suggests that while NRG3 is limiting in the process of placode formation, the ability of an individual ErbB receptor to form a heterodimer with the other family members provides sufficient signaling to allow embryonic placode formation.

The IGF/IGF1R axis has an essential role in embryonic bud development. Intriguingly, it was observed that embryos deficient in P190-B, a member of the RhoGTPase activating (RhoGAP) family that interacts with integrins, had smaller mammary buds. The defect was in both compartments, because the epithelial bud proliferation was lower and the underlying mesenchyme was aberrant. A similar phenotype was observed in embryonic mammary buds lacking IRS-1/2 (Heckman et al. 2007). Taken together, the results suggest that IGF1/IGF1R signaling through a RhoGAP and the IRS effector proteins has an essential role in the epithelial mesenchymal interactions needed for proper mammary bud morphogenesis.

Ductal Outgrowth and Differentiation

The rudimentary epithelial tree in the mammary fat pad remains quiescent until puberty onset when ovarian steroid hormone production commences and induces ductal outgrowth. Bulbous structures on the tips of the ducts, the terminal end buds (TEBs) are highly proliferative and penetrate into the fat pad as the ducts elongate. TEBs bifurcate and secondary branches form during this process, until the entire fat pad is filled with a network of branched ducts. During repeated estrous cycles the ductal network increases in complexity and side-branches grow under progesterone control. Under control of prolactin, alveolar structures bud off the ductal system during pregnancy, and these differentiate into milk-producing structures. Steroid hormones, growth hormone (GH), and prolactin are the master regulators of mammary growth and pregnancy-induced differentiation, whereas peptide factors of the EGF and FGF family, and IGF-1 have specific roles during these processes.

During ductal outgrowth multiple FGFs: FGF1, FGF2, FGF4, FGF7, and FGF10, as well as FGFR1 and FGFR2 are expressed (reviewed in Schwertfeger 2009). TEBs have high levels of FGFR2, which is essential at this stage because FGFR2 null glands penetrate the fat pad more slowly and show fewer branch points compared with WT controls (Lu et al. 2008). Indeed, it has been shown that the epithelial specific FGFR2-IIIb isoform is responsible for ductal outgrowth (Parsa et al. 2008). During pregnancy and lactation some FGFs are expressed (Coleman-Krnacik and Rosen 1994), however, to our knowledge a specific role for the FGF/FGFR signaling network at this developmental stage has not been described.

The pituitary GH is essential for pubertal mammary development (reviewed in Hovey et al. 2002); IGF1, which is produced in the mammary gland, acts as the local effector of GH (reviewed in Wood et al. 2000; Kleinberg and Ruan 2008; Kleinberg et al. 2009). During postnatal development of the gland, IGF1 is produced in both stromal and epithelial cells (Loladze et al. 2006) and, in its absence, TEBs fail to develop correctly and the glands show limited outgrowth potential (Ruan and Kleinberg 1999). These results, together with data showing that embryonic IGF1R null mammary buds transplanted into WT hosts also possess limited outgrowth potential and show defects in TEB proliferation (Bonnette and Hadsell 2001), illustrate the importance of the IGF1 axis for mammary development. The RhoGAP, p190-B mentioned previously, also modulates the IGF1/IGF1R axis during pubertal outgrowth. In p190-B heterozygous females the ductal outgrowth rate is slower and decreased levels of IRS-1/2 were observed in TEBs (Chakravarty et al. 2003). Interestingly, pregnancy hormones partially overcome the proliferation defect of the IGF1R null epithelium, potentially due to increased insulin sensitivity (discussed in Bonnette and Hadsell 2001). IGF1 is also required for lobuloalveolar development of the mammary gland (Loladze et al. 2006).

Multiple EGF-family ligands and all of the ErbB receptors are expressed in the mammary gland during ductal outgrowth, pregnancy-

induced alveolar differentiation, and in lactation (Schroeder and Lee 1998). EGF-family ligands are produced as transmembrane precursors that on cleavage bind and activate ErbB receptors. Members of the ADAM (a disintegrin and metalloproteinase) family of proteases, as well as matrix metalloproteinases (MMP) cleave and release EGF-related ligands (Sanderson et al. 2006; Bergers and Coussens 2000). During pubertal outgrowth EGFR is strongly expressed in TEBs and adjacent stroma (Coleman et al. 1988) and *waved-2* mutants with a kinase-impaired EGFR show less outgrowth (Sebastian et al. 1998). Reciprocal transplant experiments between WT epithelium and EGFR mutant stroma and the reverse, revealed that stromal EGFR is required during this phase (Wiesen et al. 1999).

The evidence pointing to epithelial-produced AR as the EGFR activating ligand at this developmental stage is very strong. Expression of AR, but none of the other EGFR ligands, is very high during puberty (Sternlicht et al. 2005). Moreover, AR appears to be a direct target of estradiol since upon injection of the steroid into 3-wk-old ovariectomized mice, AR expression is strongly up-regulated (Ciarloni et al. 2007). Finally, AR null epithelial grafts do not grow in WT mammary fat pads (Ciarloni et al. 2007). Interestingly, ADAM17 null mice have a phenotype very similar to that of AR null mice and it was shown that in the absence of this protease there is impaired cleavage and release of AR (Sternlicht et al. 2005). Thus, AR is required specifically downstream from estradiol in the epithelial compartment, where it is released by ADAM17 and acts on stromal EGFR to promote ductal elongation during puberty. This suggests a model whereby stromal cells respond to AR and produce soluble factors that act back on the epithelium. Some interesting candidates are expressed at this developmental stage including FGFs, IGF1, and HGF. Because absence of the epithelial FGFR2-IIIb also impairs pubertal outgrowth (Parsa et al. 2008), it is tempting to speculate that stromal-produced FGFs, e.g., FGF7 or FGF10, are the ligands acting on the epithelium. Indeed, it has been shown that FGF2 acts downstream from EGFR and

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supports growth of EGFR null mammary organoids in culture (Sternlicht et al. 2005). Despite the fact that stromal ErbB2 is also active at puberty (Sebastian et al. 1998), analyses carried out on ErbB2 null mammary epithelium showed that the receptor has a limiting role in the epithelium, not in the stroma. In the absence of epithelial ErbB2, impaired ductal elongation and branching (Jackson-Fisher et al. 2004; Andrechek et al. 2005), as well as alterations in TEB structures were observed (Jackson-Fisher et al. 2004) during pubertal outgrowth. ErbB2 requires a heterodimerization partner for ligand-induced activation and likely candidates for this role are NRG1 and ErbB3. On the one hand, implantation of NRG1 slow-release pellets promoted ductal outgrowth (Jones et al. 1996). On the other hand, transplantation of mammary buds from ErbB3 null embryos into recipient WT fat pads showed that in the absence of ErbB3 pubertal outgrowth was severely impaired and TEBs showed increased apoptosis (Jackson-Fisher et al. 2008).

EGFR, ErbB2, and ErbB3 each have proliferative and survival roles during mammary outgrowth. In contrast, ErbB4 has an essential role late in pregnancy and in lactation (reviewed in Stern 2008). Initial studies showed that during lactation ErbB4 was strongly phosphorylated on tyrosine (Schroeder and Lee 1998), indicative of a role at this developmental stage. A direct examination of ErbB4 null mammary epithelium showed that pubertal outgrowth was normal, however, alveolar differentiation and lactation were impaired (Tidcombe et al. 2003). Moreover, the defect was shown to be due to impaired phosphorylation of the transcription factor Stat5, which has an essential role in mammary gland differentiation and is activated during lactation by ErbB4 (Jones et al. 1999). ErbB4 is the only member of the family that undergoes γ -secretase cleavage, which releases the intracellular cytoplasmic domain of the receptor, allowing its nuclear entry (Ni et al. 2001; Sardi et al. 2006). This is interesting in light of the finding that in lactating mammary glands this domain of ErbB4 is present in the nucleus (Long et al. 2003). The other three ErbB family members have also

been detected in the nucleus of cells in various normal tissues, although this has not been described in the mammary gland (reviewed in Wang and Hung 2009).

There are two candidates for the ligand activating ErbB4 during late stages of pregnancy and in lactation. One is NRG α 1, which when deleted has a transient effect on alveolar development late in pregnancy and early in lactation (Li et al. 2002). In addition, there is evidence suggesting that HB-EGF might mediate ErbB4 activation during lactation. The heparin-sulfate proteoglycan CD44 has been shown to bind MMP-7 and pro-HB-EGF on the surface of mammary epithelial cells and this binding is required for HB-EGF processing. A lactation defect has been observed in CD44 null females. Moreover, neither HB-EGF processing, nor ErbB4 activation were observed in mammary glands lacking the proteoglycan (Yu et al. 2002).

RTKs AND MAMMARY CANCER

A prevailing theme during the proliferative phase of mammary gland development is paracrine receptor activation by ligands produced in other cells. One of the most striking differences between normal development and cancer is the ability of the estrogen receptor (ER) positive cancer cells to proliferate; in the normal gland the ER positive cells do not divide but respond by producing paracrine factors that act on other cells, AR being a good example. This characteristic appears to be lost in cancer, something that distinguishes the normal mammary gland from breast cancer (Anderson and Clarke 2004). In this section we will briefly review cancer roles for the peptides discussed in normal development.

FGFs AND FGFRs IN CANCER

The association between FGF and mammary cancer was first established in MMTV-induced tumors, where proviral insertional mutagenesis led to FGF3 transcriptional activation (Peters et al. 1989). A recent large-scale screen revealed that the FGF pathway was activated in >65% of MMTV-induced tumors. Integration sites were

adjacent to *FGFR1* and *FGFR2* in addition to *FGF3*, 4, 6, 8, and 10 (Theodorou et al. 2007). Interestingly, *FGF8* and *FGF10*, ligands for *FGFR2* (Fig. 2), are also overexpressed in human breast cancer (Marsh et al. 1999; Theodorou et al. 2004).

FGFR1, *FGFR2*, and *FGFR4* have each been implicated in breast cancer development. The 8p11-12 amplicon harboring *FGFR1* has been detected in 8%–10% of breast cancers (Adnane et al. 1991; Ray et al. 2004; Reis-Filho et al. 2006) and correlates with poor outcome in patients with ER positive tumors (Elsheikh et al. 2007), suggestive of a role in endocrine resistance. *FGFR1* is overexpressed in some, but not all tumors with the amplicon, likely reflecting the complexity of this amplicon. Irrespective of this, elevated levels of the receptor have been detected in lobular breast carcinomas (Reis-Filho et al. 2006; Xian et al. 2009). Genome-wide screens aimed at uncovering breast cancer associated genes identified single-nucleotide polymorphisms (SNP) in introns of *FGFR2* (Easton et al. 2007; Hunter et al. 2007). One of the SNPs alters the binding of Oct-1/Runx2 to the DNA and, using an in vitro transcriptional assay it was shown that this SNP increased *FGFR2* transcription (Meyer et al. 2008). Primary tumors with this SNP show elevated *FGFR2* levels (Nordgard et al. 2007). *FGFR2* amplification (10q26) has also been detected in a subgroup of triple negative (negative for ER, PR, and high *ErbB2*) breast cancers (Turner et al. 2010). High levels of the *FGFR4Arg388* allele have been correlated with poor response to chemotherapy (Thussbas et al. 2006) and endocrine therapy (Meijer et al. 2008). Introduction of this allele into a transgenic mammary tumor model led to more rapid tumor development and tumor progression (Seitzer et al. 2010). Other clinical observations on the ligands and receptors are presented in Figure 3.

EGF-FAMILY PEPTIDES AND *ErbB* RECEPTORS IN CANCER

In contrast to the FGFs that are often activated by MMTV insertional mutagenesis (Theodorou

et al. 2007), we are not aware of any reports showing that EGF-family peptides or their receptors are activated by the provirus. However, mammary transgenic models, most driven by the MMTV promoter, have been made with multiple peptides and with the EGFR and *ErbB2*. MMTV-NRG transgenics show persistent TEBs and mammary cancers arise with a long latency (Krane and Leder 1996). MMTV-EGFR transgenics develop hyperplasias and cancer (Brandt et al. 2000). Mammary tumors arising from transgenic expression of TGF α and *ErbB2*/Neu have been extensively analyzed (reviewed in Cardiff and Wellings 1999; Mikaelian et al. 2004). Moreover, MMTV-*ErbB2*/Neu mice are often used to test the importance of particular proteins in tumor outgrowth and metastasis formation (White et al. 2004; Lahlou et al. 2007).

A vast body of evidence suggests that the EGF-family peptides and their receptors have an important role in human breast cancer. *ERBB2* gene amplification was the first consistent genetic alteration found in approximately 25% of primary tumors (Slamon et al. 1987; van de Vijver et al. 1987; Berger et al. 1988). Because this was discovered more than 20 yr ago, numerous studies on the role of this receptor in breast cancer and on its potential as a therapeutic target have been published (reviewed in Hynes and Lane 2005). The basal-like subtype of breast cancer, also referred to as triple negative due to lack of ER, PR (progesterone receptor), and *ErbB2*, has a very aggressive clinical behavior (Nielsen et al. 2004). EGFR is expressed at moderate to high levels in 30%–60% of basal-like breast cancer (Reis-Filho and Tutt 2008; Thike et al. 2009). Clinical trials testing the potential of EGFR tyrosine kinase inhibitors are ongoing in breast cancer (see ClinicalTrials.gov).

IGF-1 AND IGF1R IN CANCER

There are numerous studies suggesting that IGF-1 and its receptor have important roles in breast cancer (Sachdev and Yee 2001; Pollak et al. 2004). The IGF/IGF1R axis impacts on many aspects of the tumor via its ability

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	MMTV integration site	Deregulation in human breast cancers
FGF4		
FGF4	+(1)	located in the 11q13 amplicon no data about expression or amplification
FGF6	+(1)	
FGF7		
FGF3	+(2)	located in the 11q13 amplicon found amplified in ~18% of breast cancers ^(5, 6, 7)
FGF7		Expressed in breast cancers ^(8, 9)
FGF10	+(3)	elevated expression in ~10% of breast cancers ⁽³⁾
FGF8		
FGF8	+(4)	expressed in normal breast and in breast cancers (50% of samples) higher expression than in normal breast ^(10, 11)
FGFR1		located in the 8p11-12 amplicon amplified in ~10% of breast cancers ^(12, 13) potential target in lobular breast carcinoma ⁽¹⁴⁾
FGFR2	+(1)	located in the 10q26 amplicon amplified in 4–12% of breast cancers ⁽¹²⁾ SNPs are associated with breast cancer risk ^(15, 16)
FGFR4		high FGFR4 expression predicts poor response to endocrine therapies ⁽¹⁷⁾ SNP is associated with decreased survival and resistance to adjuvant chemotherapy ^(18, 19)

Figure 3. Deregulation of FGF ligands and receptors in mammary cancer. MMTV proviral insertional mutagenesis led to the identification of five FGF ligands and the FGFR2 whose expression promoted mammary cancer. Some of these ligands have also been implicated in human breast cancer. FGFR1 and FGFR2 have also been shown to contribute to breast cancer development, whereas FGFR4 has been implicated in therapy response. The references used for this figure are: (1) (Theodorou et al. 2007), (2) (Peters et al. 1989), (3) (Theodorou et al. 2004), (4) (MacArthur et al. 1995), (5) (Naidu et al. 2001), (6) (Fioravanti et al. 1997), (7) (Berns et al. 1995), (8) (Jacquemier et al. 1998), (9) (Bansal et al. 1997), (10) (Zammit et al. 2002), (11) (Marsh et al. 1999), (12) (Adnane et al. 1991), (13) (Ray et al. 2004), (14) (Reis-Filho et al. 2006), (15) (Hunter et al. 2007), (16) (Easton et al. 2007), (17) (Meijer et al. 2008), (18) (Bange et al. 2002).

to act on the Ras/MAPK/ERK proliferative pathway and the PI3K survival pathway. Indeed, even the ratio of IGF-1 to its binding protein IGFBP-3 has been shown to modify breast cancer risk in BRCA mutation carriers (Mitchell et al. 2006). It is also interesting to note that down-regulation of IGFBP-3 leading to up-regulation of IGF1-R signaling and PI3K pathway activation was described as a mechanism underlying resistance to an ErbB

inhibitor (Guix et al. 2008). This shows not only the importance of IGF-1/IGF1R signaling in activating the PI3K pathway, but also suggests that in the clinic up-regulation of this pathway should be monitored when ErbB blocking inhibitors are used. Recently, a mouse model of basal-like breast cancer that is dependent on IGF1R has been described and in the future should be useful for testing targeted therapies (Klinakis et al. 2009).

CYTOKINES AND THEIR RECEPTORS IN NORMAL DEVELOPMENT AND CANCER

Cytokines comprise a large family of soluble proteins that mediate cell communication and induce potent biological responses often by inducing the expression of receptors, co-receptors, or adaptors. Their receptors can be grouped into families that share similar structures and signal through the Jak/Stat pathway. Notably, unlike the EGF, FGF, and IGF families of receptors, cytokine receptors do not have intrinsic kinase activity. They rely instead on receptor-associated Jak kinases (Haan et al. 2006). In mammals there are four Jaks that are ubiquitously expressed with the exception of

Jak3. On ligand binding, the Jaks transphosphorylate each other and the receptor chains to create docking sites for Stats that are then also tyrosine phosphorylated by the Jaks to create active Stat dimers. These subsequently translocate to the nucleus, bind to palindromic motifs (TTCCNGGAA), and activate transcription of sets of target genes that are specific for each Stat (Fig. 4).

CYTOKINE RECEPTOR FAMILIES

There are five main receptor families that have different receptor chains (usually two or three) and are activated by different ligands: the

Figure 4. Cytokine signaling through the Jak/Stat pathway in mammary gland development. Sequential activation of Stat6 and Stat5 control alveologenesis during pregnancy. These Stats are activated in response to specific ligands, IL-4 and IL-13 (Stat6) and Prl (Stat5) that bind to receptors of the cytokine family. During involution, cell death is mediated by Stat3, which is activated by LIF and then by OSM, which share the common gp130 receptor. Stat signaling is negatively regulated by the family of SOCS proteins that are direct transcriptional targets of each Stat factor. Once activated, Stats bind to similar consensus sequences but activate transcription of distinct sets of target genes to control mammary gland development.

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interferon (IFN), gp130, γ C, IL-3, and single chain families (Schindler and Plumlee 2008). The corresponding ligands are diverse and include the interleukins (IL), leptin, leukemia inhibitory factor (LIF), and prolactin (Prl). Different combinations of JAKs associate with each receptor, for example, Jak1 and Jak2 bind to the IFN γ receptor. The exception is the single chain family where the receptor is a homodimer and Jak2 is invariably the associated kinase. The most relevant member of this family is the Prl receptor (PRLR).

THE STAT FAMILY OF TRANSCRIPTION FACTORS

There are seven mammalian Stats (Stats1–6, 5a, and 5b) that are approximately 91 kDa in size and are distributed in pairs on three different chromosomes. They appear to have arisen from a single primordial gene with the Stat3 and Stat5a/b pair being most closely related to homologs found in *Dictyostelium*, *Caenorhabditis elegans*, and *Drosophila* (Kisseleva et al. 2002).

A variety of biochemical and genetic studies have revealed which Stats are downstream effectors of particular cytokines. Although Stat2 is specific for IFN-I, Stat4 for IL-12, IL-23, and IL-27, and Stat6 for IL-4 and IL-13, a much wider range of cytokines signal through Stat1, Stat3, and Stats5a/b. The IL-6 family (IL-6, IL-11, IL-31, LIF, CNTF, CLC/CLF, NP, CT1, and OSM), the IL-10 family (IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26), and G-CSF, Leptin, and IL-21 all activate Stat3 and the IL-3 family (IL-3, IL-5, and GM-CSF), the single chain family (GH, Prl, Tpo, and Epo), and the γ C family (IL-2, IL-7, IL-9, IL-15, and IL-21) activate Stat5a and Stat5b that are 96% identical at the amino acid level. Apart from the response to Prl (Stat5a) and GH (Stat5b) these Stats are not entirely functionally redundant and, interestingly, appear to bind DNA differently as Stat5a preferentially forms tetramers whereas Stat5b favors dimers (Verdier et al. 1998). Stat1 is the only Stat downstream from IFN γ but is also activated by some cytokines of the gp130 family and EGF.

THE ROLES OF CYTOKINES AND STATS IN MAMMARY GLAND DEVELOPMENT

Development of the embryonic mammary gland proceeds unperturbed in mice that are deficient for Stats 1, 2, 4, 5, and 6. Thus, these Stats are unlikely to be important in mammary stem cells or early tissue development. Because deletion of Stat3 results in early embryonic lethality, it has not been possible to determine whether Stat3 is important at this stage of development. However, given that Stat3 is implicated in self-renewal of embryonic stem cells in culture, it would be interesting to determine if Stat3 has a role in establishing or maintaining embryonic mammary stem cells.

The adult mammary gland is quite different and, individual members of the Stat family have important roles. Stats 1 and 3 are constitutively expressed but Stat1 is tyrosine phosphorylated only in virgin gland and in late involution, following remodeling of the gland, while Stat3 activity is highest on the day of birth and during the first 6 days of involution (Watson 2006). Stat6 is expressed also throughout adult mammary gland development, and is activated during pregnancy before Stat5. Thus, there are discrete windows of Stat activity in the adult mammary gland. The expression of cytokines is less well known although a role for LIF in ductal elongation during puberty has been described (Kritikou et al. 2003).

Genetic studies in mice have revealed roles for Stats 3, 5a and b, and 6 in the adult gland during a pregnancy cycle whereas ablation of Stat1 has not been reported to have a major effect. Stat6 is activated by the type-2 cytokines, IL-4 and IL-13, in T cells and is associated with changes in chromatin structure and transcriptional up-regulation of GATA-3, c-Maf, and NFAT1/NFATc2. Surprisingly, in mammary gland, Stat6 is also activated by IL-4 and IL-13 and this correlates with the expression of IL4R α and GATA-3 in the epithelium (Khaled et al. 2007). Importantly, in the absence of either Stat6 or IL-4 and IL-13, lobuloalveolar development is substantially delayed with numbers of alveoli reduced by about 70% although this defect is resolved by late pregnancy when

it is likely that levels of GATA-3 are sufficient to specify and maintain the lineage in the absence of Stat6. GATA-3 is critically important as demonstrated by two similar studies in which *Gata3* was conditionally deleted at different stages of mammary development (Kouros-Mehr et al. 2006; Asselin-Labat et al. 2007). Deletion of *Gata3* in alveolar cells during pregnancy resulted in a block in alveolar differentiation and failed lactogenesis (Asselin-Labat et al. 2007). Doxycycline-inducible Cre-mediated deletion of *Gata3* in late pregnancy resulted also in the disruption of ductal architecture (Kouros-Mehr et al. 2006). Using FACS to isolate subpopulations of epithelial cells revealed that the size of the luminal progenitor pool increases significantly in *Gata3*-null mice. Because overexpression of GATA3 in null cells induced the expression of the milk proteins β -casein and WAP, in the absence of lactogenic hormone stimulation, it seems reasonable to conclude that GATA-3 promotes the differentiation of lineage-restricted progenitor cells (Asselin-Labat et al. 2007).

Levels of GATA-3 are high also in the mammary epithelium of virgin mice and it is one of the earliest transcription factors to be expressed in mammary gland, being detectable in the embryonic placode. The importance of GATA-3 is illustrated further by the failure to form TEBs and a severe reduction in ductal outgrowth in the absence of GATA-3. A transcriptional link between GATA-3 and FOXA1 was uncovered using microarray and promoter analysis coupled with chromatin immunoprecipitation to identify a functional GATA-3 binding site in the FOXA1 promoter. Interestingly, FOXA1, is required for ER α transcriptional activity. Thus, GATA-3 is required at various stages of mammary gland development, placing this transcription factor earlier in the luminal lineage than Stat5 and Stat6.

It is interesting that phosphorylated Stat5 levels are reduced in Stat6-deficient mammary glands. Whether this is a consequence of reduced levels of IL-5, which is known to activate Stat5, rather than Prl is not known. A hierarchy of signaling from IL-4/IL-13 through Stat6 and GATA3 is thus an important constituent

of commitment to the alveolar luminal lineage. Notably, GATA3 is highly expressed in breast cancers of the luminal A subtype, which also express ER α (Sorlie et al. 2003).

Stats 5a and 5b have long been known to be essential mediators of lobuloalveolar development (Miyoshi et al. 2001; Cui et al. 2004). These two Stat5 gene have different roles reflecting their upstream activators Prl (Stat5a) and GH (Stat5b) although they can compensate for each other, as shown by the rescue of impaired alveologenesis and failed lactation in the Stat5a knockout mouse by Stat5b (Liu et al. 1998). It can be concluded that Stats5a and b are essential for normal differentiation and survival of alveolar mammary epithelial cells but that because ductal morphogenesis is unaffected in their absence, this requirement is specific to the alveolar lineage. This distinguishes Stat5 from GATA-3, which is required in both ductal and alveolar lineages. Recently, transplantation studies of FACS-sorted cells suggested that alveolar progenitor cells could not be generated from a presumptive earlier progenitor in the absence of Stats5a and b. Notably, whereas GATA-3 expression is unperturbed in Stat5a/b null cells, the expression of Elf5 is severely diminished (Yamaji et al. 2009).

Once again, there are parallels with the hematopoietic system because Stat5a/b have been shown to control stem and progenitor cell fate and loss of a single allele of Stat5a/b resulted in increased HSC cycling and depletion of the long-term HSC pool (Wang et al. 2009). Furthermore, results obtained with mice completely null for Stat5a/b reveal that these factors are required for the normal development of all lymphoid cells (Yao et al. 2006).

Taken together, the data suggest that there are at least two alveolar lineages: a Prl-Stat5 controlled lineage where Elf5 is an essential regulator and a Stat6-GATA-3 lineage that may be ER α positive. It is interesting that these lineages are not independent of each other because ablation of either Stat5 or GATA-3 results in a loss of differentiated epithelium perhaps indicating essential interactions between cells of these lineages. Because GATA-3 is expressed in virgin gland whereas Stat6 is activated in only a few

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ductal cells in the virgin, it may be that GATA-3 is switched off in a subset of alveolar cells during pregnancy to be replaced by Elf5.

Stat3 is essential for the initiation of apoptosis and remodeling following forced weaning (Chapman et al. 1999). In the absence of Stat3, involution is dramatically delayed and the reversible phase can be extended for up to 6 d (Humphreys et al. 2002). Stat3 is activated by a number of cytokines. In involuting mammary gland, the upstream ligand is LIF, shown using LIF deficient mice (Kritikou et al. 2003) or by implanting LIF pellets into lactating mammary glands (Schere-Levy et al. 2003). Another upstream regulator of Stat3 activity in involution is TGF β 3 (Nguyen and Pollard 2000). Interestingly, IL-6 does not regulate Stat3 activity in involution (Zhao et al. 2002) although it does in breast cancer cells, suggesting perhaps that different signals downstream from their cognate receptors specifies either a proliferative or death response to specific Stat3-activating cytokines. Recently, it has been shown that the receptor for oncostatin M (OSMR) is required for the activation of Stat3 during the second phase of involution (Tiffen et al. 2008) where it may substitute for LIF which peaks at 12 hours involution. Because OSMR is transcriptionally regulated by Stat3, this provides positive feedback on the pathway enhancing the Stat3 signal in the second phase (Tiffen et al. 2008).

Thus, cytokines and their Stat targets have important roles in lineage commitment, survival, and death of mammary gland epithelium.

CYTOKINE SIGNALING AND BREAST CANCER

As mentioned earlier, GATA-3 expression is strongly correlated with the Luminal A subtype of breast tumors, which have the best prognostic outcome (Sorlie et al. 2003). Low GATA-3 expression is an indicator of poor clinical outcome. In mouse models of breast cancer, GATA-3 suppresses tumor dissemination and maintains differentiation.

Several Stats, and in particular Stat3, have been shown to be constitutively activated in a

wide range of blood and solid tumors and are frequently activated in breast carcinoma (Watson and Miller 1995) (Fig. 5). The apparent paradox between the death-inducing function of Stat3 in involution and its oncogenic and survival function in breast tumors, has prompted extensive investigation. Apart from the different cell types involved, namely fully differentiated alveolar cells in involution compared with ductal cells in most breast carcinomas, Stat3 activation results in the secretion of cytokines. Thus, chronic Stat3 activity in tumor cells results in paracrine signaling to cells in the tumor microenvironment including infiltrating immune cells. It is well established that inflammation has tumor-promoting potential and active Stat3 is often found at the invasive edge of tumors, adjacent to inflammatory cells (Bromberg and Wang 2009). Stat3 is also a key mediator of anti-inflammatory responses downstream from the IL-10 receptor and has recently been shown to enable tumors to evade the immune system by inhibiting the maturation of dendritic cells and macrophages (Wang et al. 2004). Accordingly, inhibition of Stat3 in macrophages induced an antitumor immune response in a rat model of breast cancer (Sun et al. 2006). Recently, using an activated ErbB2 mouse model of breast cancer, it was shown that although Stat3 does not affect the initiation of tumors, it dramatically increases lung metastasis (Ranger et al. 2009). Ablation of Stat3 in this model was associated with a reduction in inflammatory and angiogenic responses. Thus, breast cancers with active Stat3 condition other cells in the tumor microenvironment by secreting cytokines that promote both metastasis and immune evasion.

Recently, a role for IL-6 in self-renewal of breast cancer stem cells was proposed (Sansone et al. 2007). Using the so-called mammosphere assay, where stem cells and their derivatives are cultured in floating spheroids, it was shown that breast tumor cells express higher levels of IL-6 mRNA than nontumor cells from the same patient although secretion of IL-6 by these cells was not measured. Furthermore, IL-6 treatment promoted spheroid growth in a Notch-3-dependent manner. IL-6 has been shown also to

Figure 5. Constitutive Stat3 activity in tumors affects the surrounding microenvironment. Chronic Stat3 activation in tumor cells results in secretion of IL-1 β , IL-6, and IL-10; these in turn act on nontumor cells, T helper 17 cells (Th17) and tumor associated macrophages (TAM) resulting in further secretion of cytokines, which act in a feedback loop to promote growth and differentiation of the tumor cells. Stat3-directed secretion of IL-10 by the tumor cells also results in inhibition of antitumor immunity, for example by inhibiting the maturation of dendritic cells (DCs). Thus, Stat3 and the cytokines that it induces, promote tumor invasion and metastasis. Diagram adapted from Pensa et al. (2009).

be a potent growth factor in ER α positive breast tumors (Sasser et al. 2007). Overexpression of IL-6 in MCF-7 breast cancer cells repressed E-cadherin expression and induced an EMT phenotype, and xenografts of these cells showed increased proliferation and advanced tumor grade (Sullivan et al. 2009). Interestingly, it has been shown recently that EMT induces stem cell-like properties (Mani et al. 2008). Cooperation between Stat3 and EGFR has been shown to be involved in EMT by mediating the expression of the TWIST gene (Lo et al. 2007). So, Stat3 could influence tumor spread by directly inducing EMT via up-regulation of TWIST expression and down-regulation of E-cadherin levels and enhancing self-renewal properties.

Stat3 and Stat5 have quite different roles in normal mammary gland development and

have been suggested to play opposing roles in cell fate with Stat3 being pro-apoptotic and Stat5 prosurvival (Clarkson et al. 2006). A subset of breast tumors have both Stat3 and Stat5 constitutive activity and a recent study showed that these tumors are more differentiated than tumors with only Stat3 activity and furthermore, have more favorable prognostic characteristics (Walker et al. 2009). These data in breast tumors contrast with those in transgenic mice that develop tumors as a consequence of overexpression of either normal or activated Stat5 although these also have a differentiated phenotype (Iavnilovitch et al. 2004). Recent work has revealed that expression of constitutively active Stat5a suppresses the motility and invasiveness of MCF7 cells while in contrast, constitutively active Stat5b has no effect suggesting that expression of Stat5a, but not Stat5b,

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could be an indicator of better prognosis in breast cancer (Tang et al. 2010).

Although there is little evidence that Stat6 is oncogenic, Stat6 deficient mice are significantly more resistant to tumors arising from 4T1 mammary adenocarcinoma cells compared with wild-type BALB/c mice (Jensen et al. 2003). Furthermore, ablation of Stat6 results in enhanced survival of mice in which 4T1 primary tumors have been surgically excised (Ostrand-Rosenberg et al. 2002). This has been proposed to result from the polarization of the immune response toward a type 1 cytokine profile because Stat6 KO mice are unable to generate a type 2 response.

Thus, the Stats that are important for normal alveologenesis in mammary gland are also important in tumorigenesis either by promoting tumor growth (Stat5), generating an inflammatory tumor microenvironment (Stat3), or by facilitating immune evasion (Stat6 and Stat3).

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