Fibroblast growth factor signalling in mouse mammary gland development

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Abstract

Fibroblast growth factors (Fgfs) and their receptors are important intercellular signalling molecules involved in many aspects of animal development. The aberrant expression of the Fgfs or the inappropriate activation of their cell surface receptors have been implicated in tumorigenesis. Here, we describe the evidence that as well as playing a critical role in the formation of the mammary primordia during embryogenesis, signalling by Fgfs is necessary for optimal lobuloalveolar development of the mouse mammary gland during pregnancy.

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Introduction

The mouse mammary gland has been extensively studied as a model for breast cancer, as well as a system to investigate the mechanisms underlying branching morphogenesis (reviewed in Cunha & Hom 1996, Daniel & Smith 1999, Robinson *et al.* 1999). Development of the mouse mammary gland is usually considered to occur in three separate stages: embryonic, postnatal and postpubertal. Embryonic development is such that by birth a very rudimentary branched ductal structure is present under the epidermis that lies adjacent to the presumptive mammary fat pad. During the 6–7 weeks of postnatal development the rudimentary ducts extend and branch into the underlying fat pad to form a simple tree-like structure. After puberty, the virgin gland may undergo small changes with the appearance of a few alveoli stimulated by the hormonal fluctuations of the oestrous cycle. However, a substantial part of mammary gland development occurs in the adult female. This can be essentially subdivided into four phases (reviewed in Daniel & Silberstein 1987). The first, which proceeds through early to mid-pregnancy is characterised by epithelial cell proliferation and results in further branching of the ductal tree and the appearance along the ducts of alveoli. During the second phase, from mid- to late pregnancy, the alveoli increase in density to form lobules, and the systemic rise in lactogenic hormones initiates their functional differentiation into milk-producing units. At parturition, the rapid drop in progesterone levels allows both synthesis and secretion of milk to reach maximum levels. Finally, after the young are weaned, the glands involute. This is characterised by

apoptosis of the epithelial component of the tissue as it remodels to a resting state similar in structure to the virgin gland. Hence the mammary gland is particularly useful for the study of branching morphogenesis and functional differentiation in tissues. The timing of pregnancy can be easily controlled. Moreover, there are ten glands that can be analysed and, importantly, the mammary gland can be physically and genetically manipulated without seriously affecting the health of the animal.

Mammary tissues show a remarkable regenerative capacity, a process that was recently demonstrated by Kordon & Smith (1998). By using genetically marked epithelial cells and serial transplantation they were able to show that a single cell could eventually generate an entire mammary gland. Hence an understanding of the properties of this potential mammary stem cell, and its more differentiated daughters may have important implications regarding the characteristics of cells that become targets for neoplastic transformation.

In mammals, fibroblast growth factors (Fgfs) are a large multigene family that transduce signals by activating the cytoplasmic tyrosine kinase of transmembrane receptors (reviewed in McKeehan *et al.* 1998, Ornitz 2000). Although their name implies that they can induce the proliferation of fibroblasts, collectively Fgfs are broad-spectrum mitogens acting on cells of ectodermal, endodermal and mesenchymal origin. They also act as cell survival factors and motogens as well as inducing or inhibiting cell differentiation depending on context (Basilico & Moscatelli 1992, McKeehan *et al.* 1998). The extracellular domain of the receptors (FgfR) for these ligands is composed of two or three Ig-like loops, with

loops II and III configured to form the Fgf-binding domain. However, signal transduction is complicated by the need for a co-receptor in the guise of a proteoglycan, where the glucosaminoglycan side chains function to form a heteromeric complex with the Fgf and FgfR (reviewed in Johnson & Williams 1993, Ornitz 2000). There are four receptor genes that encode, by means of alternative splicing, a number of different receptor isoforms. For example, *FgfR1*, *FgfR2* and *FgfR3* each encode two isoform sets that show distinct Fgf binding properties. Moreover, the different receptor isoforms are expressed in different cell lineages (Ornitz 2000). Each of these sets is designated after the isoform specific exon which it encodes, IIIb and IIIc (Fig. 1). The receptor FgfR2-IIIb is expressed on many types of epithelium, while FgfR2-IIIc occurs on cells of mesenchymal origin (Peters *et al.* 1992, Orr-Urtreger *et al.* 1993). The Fgfs that signal through the epithelial receptor FgfR2-IIIb are predominantly expressed in the adjacent mesenchyme, thereby providing the basis for instructive paracrine signalling (Finch *et al.* 1989). This process is of paramount importance during organ morphogenesis. Therefore, the Fgfs can be considered as intercellular signalling molecules capable of mediating a variety of properties and cell

behaviours. *In vivo* studies suggest these Fgfs must function in concert with other secreted factors to co-ordinate the growth and differentiation of cell masses during organ morphogenesis as recently reviewed (Hogan & Yingling 1998).

FgfR2-IIIb signalling is implicated in lobuloalveolar development

A crucial part of FgfR signal transduction is the ligand-mediated dimerisation of the receptor. This leads to an activating transphosphorylation of the kinase domain and concomitant signalling through associated second messengers. Thus if kinase deleted receptor mutants are introduced into cells that constitutively express the wild-type receptor, the latter can be sequestered into inactive heteromeric complexes with the truncated receptor. Therefore, by expressing an excess of mutant receptor in a target cell population, signalling through the wild-type receptor can be inhibited. To assess the role of Fgf signalling in postpubertal mammary gland development, dominant negative forms of two different FgfRs were expressed in the mammary epithelium (Jackson *et al.* 1997). The aim was to

Figure 1 Schematic depiction of Fgf signalling and its abrogation by dominant negative receptors. On the left is the structure of an Fgf receptor, indicating the three Ig-like loops, and the region of alternative splicing in the third Ig-loop which confers ligand specificity. In conjunction with its interaction with the glucosaminoglycan side chains of proteoglycan, ligand binding causes heteromeric complex formation, autophosphorylation and subsequent signal transduction. On the right the depiction shows how the tyrosine kinase deleted receptor is able to sequester functional receptor into inactive complexes, thus reducing signal transduction in the targeted cells.

inhibit autocrine signalling within the epithelium as well as to disrupt paracrine signalling from the mammary mesenchyme (see Fig. 1 and Jackson *et al.* 1997). As the formation of wild-type and mutant receptor dimers requires receptor interaction with ligand, dominant negative constructs were selected for their ability to bind a wide range of Fgfs. Accordingly, FgfR1-IIIc, which can be activated by Fgf1, Fgf2, Fgf4, Fgf6, Fgf8, and FgfR2-IIIb, which can be activated by Fgf1, Fgf3, Fgf7 and Fgf10, were chosen (Ornitz *et al.* 1996). These mutant receptors, lacking sequences encoding the tyrosine kinase domain, were each introduced into the mouse germ line as transgenes expressed from a mouse mammary tumor virus promoter (Jackson *et al.* 1997).

Examination of mammary glands from mice expressing a dominant negative FgfR1-IIIc receptor showed no discernible morphological changes relative to control mice, suggesting that its wild-type full length counterpart does not have an important or unique function in adult mammary gland development (Jackson *et al.* 1997). In contrast, expression of a dominant negative FgfR2-IIIb receptor in the mammary epithelium caused an inhibition of lobuloalveolar development during pregnancy (Fig. 2). This was seen as a more sparsely developed ductal tree with fewer alveoli (Fig. 2C and Jackson *et al.* 1997). Alveoli that were present differentiated to produce what appeared to be normal amounts of milk (Fig. 2G; Jackson *et al.* 1997). However, the runt appearance of the litters from these mice indicated that total milk production was nevertheless significantly reduced. This was attributable to the scarcity of alveoli in the mammary tissue. These findings are consistent with two previous observations. First, FgfR2-IIIb receptors are normally expressed on mammary epithelial cells and therefore would be subject to inhibition by a dominant negative mutation (Mathieu *et al.* 1995). Secondly, it explains why Fgf3 and Fgf7, which are normal ligands for this receptor, can act as oncogenes when inappropriately expressed in the mammary epithelium. In these circumstances the Fgf is expressed in the same cell as its receptor and therefore signals in a constitutive autocrine manner (Dickson *et al.* 1984, Muller *et al.* 1990, Stamp *et al.* 1992, Kitsberg & Leder 1996). However, when either of these two ligands are expressed as transgenes in the mammary epithelium, the resulting tumours are focal in nature and arise sporadically after several months. This indicates that other genetic changes are necessary for progression to frank neoplasia.

The inhibition of Fgf signalling by the dominant negative FgfR2-IIIb, which gives rise to inadequate lobuloalveolar development, must be mediated by Fgf(s) that are normally present in the mammary gland. It is unlikely to be Fgf1 as this ligand also binds to FgfR1-IIIc and the dominant negative form of this receptor did not act as an inhibitor of lobuloalveolar development. Fgf3 is not expressed in the mammary gland, but other potential candidates are Fgf7 and Fgf10. However, mice deficient for Fgf7 are viable and have no reported mammary phenotype (Guo *et al.* 1996), leaving

Fgf10 as the prime candidate. However, the lack of a mammary gland phenotype in the absence of Fgf7, might merely reflect functional redundancy among the Fgfs. Both Fgf7 and Fgf10 are normally expressed in mesenchymal tissue such as the dermis, so that both these growth factors would have the potential to signal to the mammary epithelium in a paracrine manner. Nevertheless, it is not clear what function such signalling would play in the adult mammary gland during pregnancy, nor is it clear how this signalling might be regulated. Fgf signalling may act to stimulate the proliferation of the mammary epithelium, contributing directly to lobuloalveolar growth, however it could also function as a cell survival stimulus. For example, this latter property of FgfR2-IIIb signalling is particularly important in early limb bud development (DeMoerloose *et al.* 2000, Revest *et al.* 2001). In the mammary gland it could serve as a mechanism to guard against unscheduled proliferation. Thus one of the main functions in the mammary gland of the Fgfs could be to prevent apoptosis during the programmed proliferation of the mammary epithelium. The latter is principally orchestrated by steroid and peptide hormones, suggesting that Fgf signalling is most likely regulated, directly or indirectly, by these systemic factors.

Embryonic mammary gland development fails in *FgfR2-IIIb*^{-/-} mice

To investigate a possible role for FgfR2-IIIb signalling in the early stages of mammary gland development, analysis of mice with a germ line inactivation of *FgfR2-IIIb* was undertaken (DeMoerloose *et al.* 2000). In the mouse, the first signs of mammary gland development appear around embryonic day 11 (E11) with five pairs of epithelial cell thickenings on the epidermis (Cunha & Hom 1996, Daniel & Smith 1999). The mammary primordia arise on the ventral skin in a region between the forelimb and hindlimb buds. These thickenings are not visible on the surface of the embryonic skin but can be detected by sectioning and histological staining of the ventral epidermis. By E14.5 the buds develop into a ball of epithelial cells joined by a stalk to the epidermis (Fig. 3). This invagination of the epithelium is accompanied by an accumulation of underlying mesenchyme. Mice deficient for *FgfR2-IIIb* do not develop discernible mammary primordia. However, interpretation of this result is complicated by an accompanying defect in epidermal differentiation found in the *FgfR2-IIIb*^{-/-} mice (Fig. 3). The skin of the mutant mice remains as a single layer of epithelium for about 1 day longer than normal, and when it eventually differentiates the epidermis is of a reduced thickness (DeMoerloose *et al.* 2000). However, mammary primordia have not been detected in these mice even after epidermal differentiation.

Figure 2 *In situ* hybridisation analysis and phenotypic consequences of expressing a dominant negative form of *FgfR2-IIIb*. *FgfR2* expression in mice expressing a dominant negative *FgfR2-IIIb* transgene (A and B). The same views under bright field (A) and dark field (B) illumination. Note the intense level of signal over the mammary epithelial cells. The fat pad and muscle components show only background staining. Control mammary glands at a similar stage gave a signal close to background, suggesting that *FgfR2-IIIb* is normally expressed at low levels (Jackson *et al.* 1997). Whole mount mammary gland preparations from 17 day pregnant (C and D) and 1 day post partum (E and F) mice. Panels C and E are from transgenic mice and D and F from control mice. Histological sections of mammary gland at 1 day post partum from transgenic and control mice (G and H respectively). In the mammary glands from transgenic mice, the alveoli while reduced in number are nevertheless distended with milk (Jackson *et al.* 1997).

Figure 3 Expression of *FgfR2* in mammary primordia and epidermis in embryos at E14.5 days. *In situ* hybridisation on sagittal sections using an *FgfR2-IIIb* probe on wild type mice (A and B), and an *FgfR2* tyrosine kinase probe on *FgfR2-IIIb*^{-/-} mutant mice (C and D). Note that the tyrosine kinase probes detect expression of the defective *FgfR2-IIIb* mRNA.

Analysis of *FgfR2-IIIb* gene expression has shown that it is present in the ectoderm and subsequently the basal layer of the skin epidermis, as well as the mammary primordium (Fig. 3 and Cunha & Hom 1996). Expression of the receptor in the epidermal basal cells and subsequently in the developing hair follicles is maintained throughout foetal development. The mesenchyme that accumulates around the mammary buds expresses *Fgf7*, a ligand for *FgfR2-IIIb* activation (Cunha & Hom 1996). Hence the distribution of receptor and ligand provides the basis for an instructive paracrine signal from the mesenchyme to the mammary primordium. As indicated above, mice deficient for *Fgf7* do not appear to suffer any mammary abnormalities, so signalling by this potential ligand is clearly not essential for mammary gland development. Recently, mice deficient for *Fgf10* have been shown to have a complex phenotype similar to that of *FgfR2-IIIb*^{-/-} mice, suggesting that *Fgf10* is the main ligand for this receptor (DeMoerlooze *et al.* 2000, Ohuchi 2000). Moreover, in both these mutant mouse strains, many organs that develop by budding or branching morphogenesis are affected. Thus, although not reported in the initial analysis, it seems likely that the *Fgf10*^{-/-} mice might also display a mammary gland defect, similar to that seen in the *FgfR2-IIIb*^{-/-} mice.

The precise role of Fgfs in embryonic mammary gland development is not clear, as these intercellular signalling molecules display many diverse biological activities and are involved in proliferation, motility, differentiation and survival of cells. *In vitro* cultures of mammary primordia suggest their development forms at least in part by cell

accretion, as few proliferating epithelial cells have been detected in the mammary bud at E13 (Robinson *et al.* 1999). Hence it is possible that initially Fgf signalling is important in the accretion of cells in the epithelium thereby inducing formation of the mammary bud. This would be consistent with the role proposed for *Fgf10* and *FgfR2-IIIb* in lung development, since in both *Fgf10*^{-/-} and *FgfR2-IIIb*^{-/-} mice the primary bronchial bud fails to develop (Min *et al.* 1998, Sekine *et al.* 1999, DeMoerlooze *et al.* 2000). In this tissue the receptor is normally present in the epithelium and the ligand is expressed in two patches in the mesenchyme where it has the potential to attract the two arms of the bifurcating bronchus. Interestingly, Fgf signalling is also involved in branching of the tracheal network in *Drosophila*. This occurs by cell movement and shape change alone; proliferation does not occur (Glazer & Shilo 1991, Reichman-Fried & Shilo 1995, Lee *et al.* 1996). These observations emphasize the conserved nature of these signalling systems throughout animal development.

The above studies on Fgfs in mammary gland suggest that they have a function in both the embryo and in the adult during pregnancy. However, the regulation of Fgf function is likely to be quite different at the two stages of development. Embryonic development of the mammary gland appears to be independent of systemic hormone action as demonstrated by explant studies of the mammary primordia (Kratochwil 1969). In contrast, the tissue changes that occur during pregnancy are controlled by oestrogen and progesterone, as well as other peptide hormones. Presumably these systemic hormones act to regulate cell function through the expression of local tissue modulators, such as the Fgfs.

As discussed above, the deregulated expression of Fgfs in the mammary gland has been implicated in mammary tumorigenesis. In mice this occurs by retroviral activation. However, the role of Fgfs in human breast cancer is more contentious (Dickson *et al.* 2000). Nevertheless, the presence of activating mutations in *FGFR3* has been associated with bladder and cervical cancers, suggesting that similar mutations might also be involved in breast cancer (Cappellen *et al.* 1999). Analysis of DNA from breast tumours for the known activating mutations may soon answer this question.

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