Journal of Cell Science 110, 1261-1268 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JCS3556

Fibroblast growth factor receptor signalling has a role in lobuloalveolar development of the mammary gland

David Jackson[1,*], Janine Bresnick[1,*], Ian Rosewell[2], Tracy Crafton[2], Richard Poulsom[3], Gordon Stamp[4] and Clive Dickson[1,†]

[1]Laboratory of Viral Carcinogenesis, [2]Transgenic animal Unit, and [3]Histopathology Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX, UK
[4]Royal Postgraduate Medical School, Hammersmith Hospital, London, UK

*The first two authors contributed equally to the work in this paper
†Author for correspondence

SUMMARY

We have used the mouse mammary tumor virus promoter to express two dominant negative (DN) fibroblast growth factor receptor (FGFR) isoforms in the mammary epithelium of transgenic mice. While expression of DN-FGFR1(IIIc) showed no discernible phenotype, a similar kinase negative form of FGFR2(IIIb) caused a marked impairment of lobuloalveolar development. The growth retardation was apparent by mid-pregnancy and persisted in the post-partum glands. Despite the substantial underdevelopment of the mammary gland there was a measurable lactational response, but it was insufficient to properly sustain the new-born pups. These findings demonstrate that fibroblast growth factor signalling is necessary for pregnancy dependent lobuloalveolar development of the mammary gland.

Key words: Fibroblast growth factor receptor, Dominant negative receptor, Mammary gland abnormality

INTRODUCTION

Post-natal development of the mouse mammary gland involves the endocrine action of steroid and peptide hormones which are believed to mediate growth and branching morphogenesis by regulating the activity of locally acting factors (reviewed by Daniel and Silberstein, 1987). During pregnancy a new program of development ensues which is characterised morphologically by the budding of alveoli from the ductal network to form lobules, the units of milk synthesis and secretion. After the young mice have weaned, the lactating gland undergoes a process of involution, which involves massive apoptosis in the lobuloalveolar epithelium with a reformation of the ductal network. This series of morphological changes is systemically regulated by steroid hormones from the ovary and peptide hormones from the ovary, pituitary and placenta. At the level of tissue remodelling, several secreted growth factors have been suggested to have a role, these include members of the EGF, TGF-β, HGF, Wnt and FGF families of growth modulators (Buhler et al., 1993; Coleman-Krnacik and Rosen, 1994; Gavin and McMahon, 1992; Jhappan et al., 1993; Pierce et al., 1993; Ruan et al., 1992; Snedeker et al., 1991; Yanmin et al., 1995). We have been particularly interested in the latter family because several members have been implicated in mammary neoplasia in mice infected with mouse mammary tumor virus (MMTV). In fact, *Fgf-3*, *Fgf-4* and *Fgf-8* were discovered as proto-oncogenes that had been transcriptionally activated by the nearby insertion of MMTV proviral DNA (reviewed by Peters, 1991; MacArthur et al., 1995). The ability of FGF3 to induce hyperplasia of the mammary epithelium was further substantiated by its targeted expression in transgenic mice (Muller et al., 1990; Ornitz et al., 1992a; Spivak-Kroizman et al., 1994; Stamp et al., 1992). Taken together, these observations suggest FGFs could play a role in normal mammary gland function.

The fibroblast growth factor family consists of several structurally related proteins that show a variety of biological properties including proliferative and neurotrophic activities, as well as tissue dependent modulation of cellular differentiation (reviewed by Basilico and Moscatelli, 1992; Burgess and Maciag, 1989). In mammals, the FGFs have been implicated in embryonic development, angiogenesis, wound healing and in some pathological conditions such as cancer and skeletal dysplasias (reviewed by Wilkie et al., 1995). FGF signalling is mediated through an interaction with high affinity transmembrane receptors exhibiting intrinsic tyrosine kinase activity (reviewed by Jaye et al., 1992; Johnson and Williams, 1993). These receptors are activated by dimerization which requires presentation of the ligand by a low affinity receptor, such as a heparan sulphate proteoglycan (Ornitz et al., 1992b; Ornitz and Leder, 1992). There are four known FGF receptor genes, designated *Fgfr1* to *Fgfr4*, which encode a cytoplasmic tyrosine kinase domain, an extracellular region composed of two (β-form) or three (α-form) immunoglobulin-like domains, depending on the choice of splice sites. *Fgfr1*, *Fgfr2* and *Fgfr3* also contain alternative exons (known as IIIb or IIIc) which encode the downstream half of the membrane proximal Ig-loop. The two Ig-loops adjacent to the membrane form the

ligand binding site, so that alternative exon usage has a profound effect on FGF binding specificity (Chellaiah et al., 1994; Cheon et al., 1994; Zimmer et al., 1993).

Several members of the FGF family and their receptors have been detected in the mammary gland (Coleman-Krnacik and Rosen, 1994). However, delineating a role for FGF signalling is confounded by the complex cellular composition of this organ. Redundancy of ligand-receptor interactions complicates the situation further. For example, FGF3 activates the IIIb isoforms of FGFR1 and FGFR2, while FGF4 stimulates the IIIc isoforms of these receptors as well as FGFR4, and FGF1 stimulates all known FGF receptors (see Ornitz et al., 1996). However, signalling can be blocked for all receptors that bind to a common ligand if one of the interacting receptors is expressed as a dominant negative. Hence, any DN-FGFR should block signalling by FGF1 since this ligand binds all known FGF receptors and could therefore sequester each receptor isoform into an inactive dimer. In contrast, FGF7 binds a single receptor isoform and should only block signalling when it interacts with a DN receptor of the same isotype (Kashles et al., 1991; Ueno et al., 1992). Recently expression of dominant-negative FGF receptors has been used to show a requirement for FGF signalling in mesoderm formation of *Xenopus* embryos (Amaya et al., 1991); as well as branching morphogenesis of mouse lung and proper tissue organisation and repair of mouse skin (Peters et al., 1994; Werner et al., 1993, 1994). We have used a similar approach to investigate the role for FGFs in mammary gland development during pregnancy and lactation by targeting kinase defective isoforms of FGFR1(IIIc) and FGFR2(IIIb) to the mammary epithelium. Here we show that expression of a kinase negative FGFR2(IIIb) receptor, but not a kinase negative FGFR1(IIIc), inhibits lobuloalveolar development of the mouse mammary gland.

MATERIALS AND METHODS

Construction of DN-FGFR1 and DN-FGFR2

A kinase negative form of FGFR1(IIIc) was constructed by ligating a 1.1 kb cDNA insert encoding an external domain composed of three Ig-loops, a transmembrane and juxtamembrane domain into pMMTV (Muller et al., 1990) to create DN-FGFR1. To facilitate cloning the cDNA ends were modified to contain a *Hin*dIII and *Eco*RI site at the 5′ and 3′ ends, respectively. To generate a kinase deleted form of FGFR2(IIIb) a cDNA encoding the two Ig-loop variant of the receptor was digested with *Eco*RI and *Xba*I to release a 1.0 kb fragment encompassing the extracellular, transmembrane and cytoplasmic juxtamembrane domains, and this was ligated into the polylinker of plasmid pGEM4. To provide a translational stop codon and a second *Eco*RI site, two complementary oligonucleotides (CTAGAGTAATCTGAGGAATTCACCTGCA and GGTGAATTCCTCAGATTACT) were annealed and inserted between the *Xba*I and *Pst*I site of the pGEM4 polylinker. The modified cDNA was then inserted as an *Eco*RI fragment into expression plasmid pMMTV to create DN-FGFR2.

Transfections and [^{3}H]thymidine incorporation assays

To isolate stable cell clones expressing dominant negative forms of the receptors, 18 μg of DN-FGFR1 or DN-FGFR2 plasmid DNA and 2 μg of pDOBS-BS plasmid (Morgenstern and Land, 1990) containing a neomycin resistance gene were co-transfected into HC11 cells using a standard calcium phosphate precipitation procedure and colonies of resistant cells selected in 200 μg/ml of G418. HC11 cells were grown in RMPI-1640 supplemented with 10% fetal calf serum, 5 μg/ml insulin and 10 ng/ml epidermal growth factor (EGF) (Ball et al., 1988). For the [^{3}H]thymidine incorporation assay, 2×10^4 cells/well were transferred to 48-well tissue culture plates in 0.5 ml of growth medium. After 24 hours, HC11 cells were made quiescent by replacing the growth medium with DMEM containing 0.1% new born calf serum. After a further 72 hours, the cells were treated for 22 hours with FGF1 (10 ng/ml) in the presence or absence of 10^{-6} M dexamethasone in fresh medium containing 0.1% serum. [^{3}H]Thymidine incorporation measurements were performed as previously described (Kiefer et al., 1993); the control cells in these experiments were HC11 cells co-transfected with the pMMTV and pDOBS-BS plasmids.

Generation of transgenic mice

Linearized fragments of DN-FGFR1 or DN-FGFR2 were separated on a 0.8% agarose gel, recovered by electroelution, and then centrifuged through a 0.2 μm Spin-X™ column. A solution containing 1-3 ng/μl, of purified DNA fragment was injected into the pronuclei of mouse zygotes obtained from mating C57BL × CBA mice. After injection they were transferred to the oviduct of pseudopregnant females (Hogan et al., 1986). Ten days after littering, mouse tail DNA was screened for the presence of the transgene by Southern blot analysis. The digest was separated on a 0.8% agarose gel, depurinated in 0.25 M HCl for 15 minutes, before transfer to Hybond N+ membranes (Amersham International) using 0.4 M NaOH as a blotting reagent. After rinsing twice in 2× SSC the membranes were hybridized with a ^{32}P-labelled MMTV-LTR probe and autoradiographed.

Analysis of tissue RNA

Total RNA was isolated from mammary glands by homogenisation in TRIzol reagent using a Polytron PT3000 (Kinematic AG, Switzerland) as described by the manufacturer (Gibco BRL). Samples of 10 μg of total RNA were separated on denaturing formaldehyde gels, transferred to membranes (Hybond N) and hybridised with a ^{32}P-labelled *Fgfr1* or *Fgfr2* probe (Mathieu et al., 1995). β-Casein RNA was detected using a cDNA probe kindly provided by Drs Jennifer Liao and Jeffery Rosen, Baylor College of Medicine, Texas. In some experiments a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe encoding the last 258 bp of coding sequence was used to monitor RNA loading (Tso et al., 1985).

Analysis of milk proteins

After extraction of RNA (see above) the remaining TRIzol reagent was processed for protein isolation according to the manufacturer's instructions. Protein concentration was measured using the Bradford assay (Pierce) and 5 μg samples of protein were fractionated by SDS-PAGE and transferred onto nitrocellulose membranes for immunoblotting. Milk proteins were detected using a rabbit polyclonal serum made against total milk proteins (generously provided by Dr Nancy Hynes, Friedrich Miescher-Institut, Basel). Antibodies to non-milk proteins present in the antiserum were absorbed by adding 1 ng/ml of protein extract from virgin mammary gland tissue directly to the primary antibody solution. Antibody binding was visualised by ECL as described by the manufacturer (Amersham International).

Histology assessment

For histological examination, tissue samples were fixed in buffered 10% formalin, dehydrated through a graded alcohol series, embedded in paraffin wax and sectioned at 4 μm before staining with haematoxylin and eosin. Mammary tissue for whole mount processing was spread on a glass slide, fixed in methanol:chloroform:acetic acid (6:3:1) and stained with carmine red as described by Banerjee et al. (1976).

In situ hybridization

The sites of DN-FGFR RNA expression in mammary tissue were

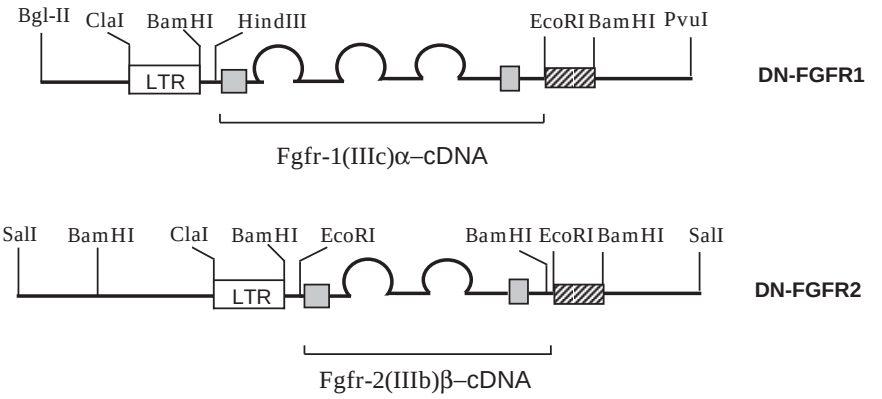

Fig. 1. Schematic depiction of the transgenes, DN-FGFR1 and DN-FGFR2. The constructs contain a 5′ truncated MMTV-LTR as the promoter (Muller et al., 1990) and were joined to the α-isoform of a mouse FGFR1(IIIc) cDNA to generate DN-FGFR1 or the β-isoform of a FGFR2(IIIb) cDNA to form DN-FGFR2. Both transgenes contain the receptor external domain, transmembrane segment and the cytoplasmic juxtamembrane region up to a naturally occurring *Bam*HI site. The shaded boxes represent the signal peptide and transmembrane domains. To the 3′ side of the receptor coding sequences is a termination codon and SV40 poly(A) addition site indicated as a hatched box.

determined using in situ hybridization with an antisense riboprobe labelled with [^{35}S]UTP (approx. 800 Ci/mmol; Amersham International) as previously described (Senior et al., 1988; Wright et al., 1990). Sections were cut from formalin fixed paraffin embedded tissues. Autoradiography was at 4°C from 6 to 11 days, after which time sections were developed, then counter stained with Giemsa.

RESULTS

Construction and testing of dominant-negative FGF receptors

DN-FGFR transgenes were constructed by deleting the kinase domain from cDNAs encoding either *Fgfr1*(IIIc)α or *Fgfr2*(IIIb)β and fusing the modified cDNA to the MMTV promoter in the expression plasmid pMMTV (Fig. 1). The MMTV promoter contains several glucocorticoid responsive elements which cause an enhanced level of transcription in the presence of dexamethasone. Using this property of the promoter, correct functioning of the transgenes was tested in cell culture. The constructs designated DN-FGFR1 and DN-FGFR2 were introduced into the mammary epithelial cell line HC11 by co-transfection with an expression plasmid which confers resistance to the drug G418. Clones containing DN-FGFR1 or DN-FGFR2 were isolated and the level of expression measured by northern blot analysis (Fig. 2A and B). In the examples shown, expression of the transgenes was detected in the transfected clones in the absence of dexamethasone but at greatly enhanced levels in its presence. To measure the effect of elevated dominant negative receptor expression on the mitogenic response to FGF1, clones expressing DN-FGFR1 or DN-FGFR2 and controls were used in a [^{3}H]thymidine incorporation assay. As shown in Fig. 2C, dexamethasone (10^{-6} M) caused a marked inhibition of [^{3}H]thymidine incorporation in the DN-receptor expressing cells but had no significant effect on the control cells. This finding shows that expression of the two dominant negative FGF receptor constructs, DN-FGFR1 and DN-FGFR2, can inhibit FGF1 induced DNA synthesis.

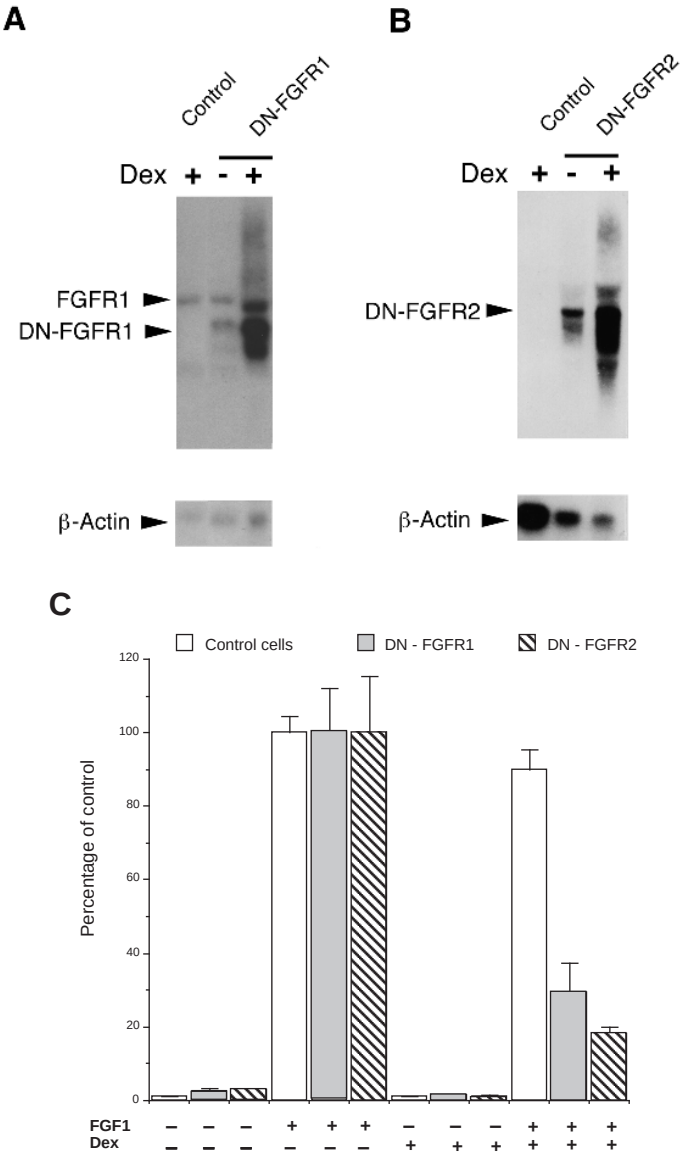

Fig. 2. Expression of dominant negative FGF receptors and the inhibition of FGF1 induced DNA synthesis. Northern blot analysis of DN-FGFR1 (A) and DN-FGFR2 (B) expression in clones of HC11 cells containing the respective transgenes as indicated and grown in the presence (+) or absence (–) of dexamethasone (10^{-6} M). The blots were re-probed with a β-actin cDNA to assess the relative RNA loading levels. (C) Histogram showing [^{3}H]thymidine incorporation into DNA following addition of FGF1 (10 ng/ml) in control cells and DN-FGFR1 and DN-FGFR2 expressing HC11 cells in the presence or absence of dexamethasone as indicated.

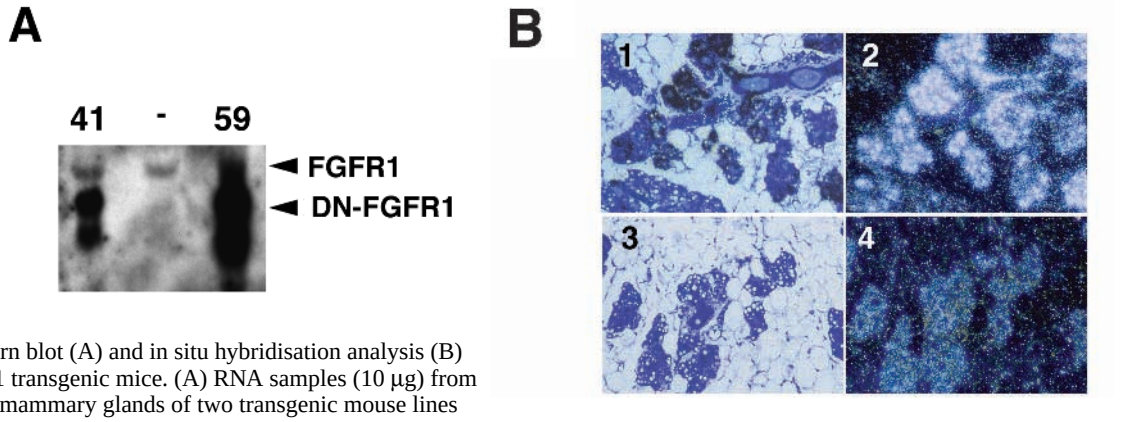

Fig. 3. Northern blot (A) and in situ hybridisation analysis (B) of DN-FGFR1 transgenic mice. (A) RNA samples (10 μg) from mid-pregnant mammary glands of two transgenic mouse lines (41 and 59) and a control gland were separated on denaturing 1% agarose gels, transferred to membranes and hybridized with a probe to *Fgfr1*. The endogenous receptor transcripts were detected as an ~4.5 kb band at much lower abundance than the major transgene band of 3.5 kb as indicated. (B) Formalin-fixed tissue sections of mid-pregnant mammary glands were hybridised with an ^{35}S-labelled *Fgfr1* antisense RNA, and counter-stained with Giemsa's stain. (1 and 3) Bright field and (2 and 4) the same views, in dark field illumination, of mid-pregnant mammary tissue from DN-FGFR1 (1 and 2) and control (3 and 4) mice, respectively.

Transgenic mice expressing DN-FGFR1

Several founder mice containing the DN-FGFR1 transgene were identified by Southern blot analysis and subsequently established as lines. Analysis of RNA from mammary tissue of the transgenic lines identified two expressing DN-FGFR1 at high level compared to the endogenous FGFR1 (Fig. 3A). To investigate sites of transgene as well as endogenous FGFR1 expression, sections of mammary gland were analysed by in situ hybridization using a probe against FGFR1. A strong positive signal was detected over the alveolar structures of the transgenic mammary gland (Fig. 3B) showing that the epithelium is the principle site of transgene expression. Tissue sections from control glands prepared in parallel show only very faint labelling over the alveolar structures, indicating a low level of FGFR1 expression, consistent with the northern blot result (Fig. 3A).

Several mammary glands from DN-FGFR1 transgenic and control mice, at different stages of pregnancy and lactation, were processed as whole mounts, or sectioned for staining with haematoxylin and eosin. For both transgenic lines expressing DN-FGFR1, the gross morphology (whole mounts not shown) and histological appearance of the mammary glands were found to be indistinguishable from those of non-transgenic control mice at all stages of pregnancy and lactation (Fig. 4A,B,C compared with D,E,F, respectively).

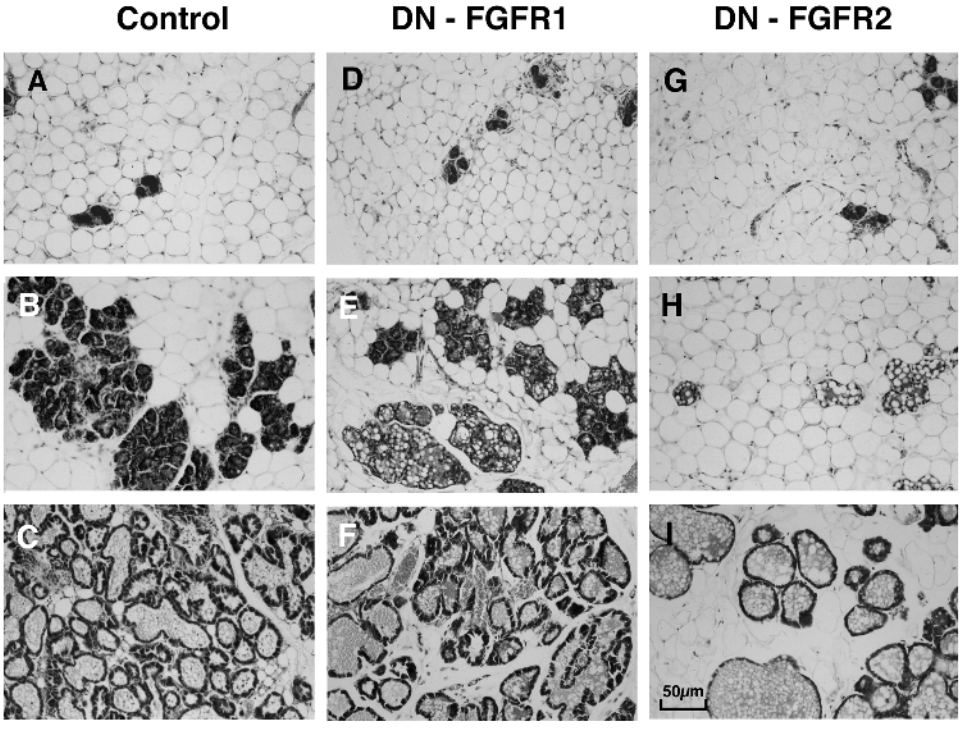

Fig. 4. Histological examination of mammary glands from DN-FGFR1, DN-FGFR2 and control mice. Sections from 8-day pregnant (A,D,G), 16-day pregnant (B,E,H) and 5-day post-partum mammary glands (C,F,I) of control (A,B,C), DN-FGFR1 (D,E,F) and DN-FGFR2 (G,H,I) mice.

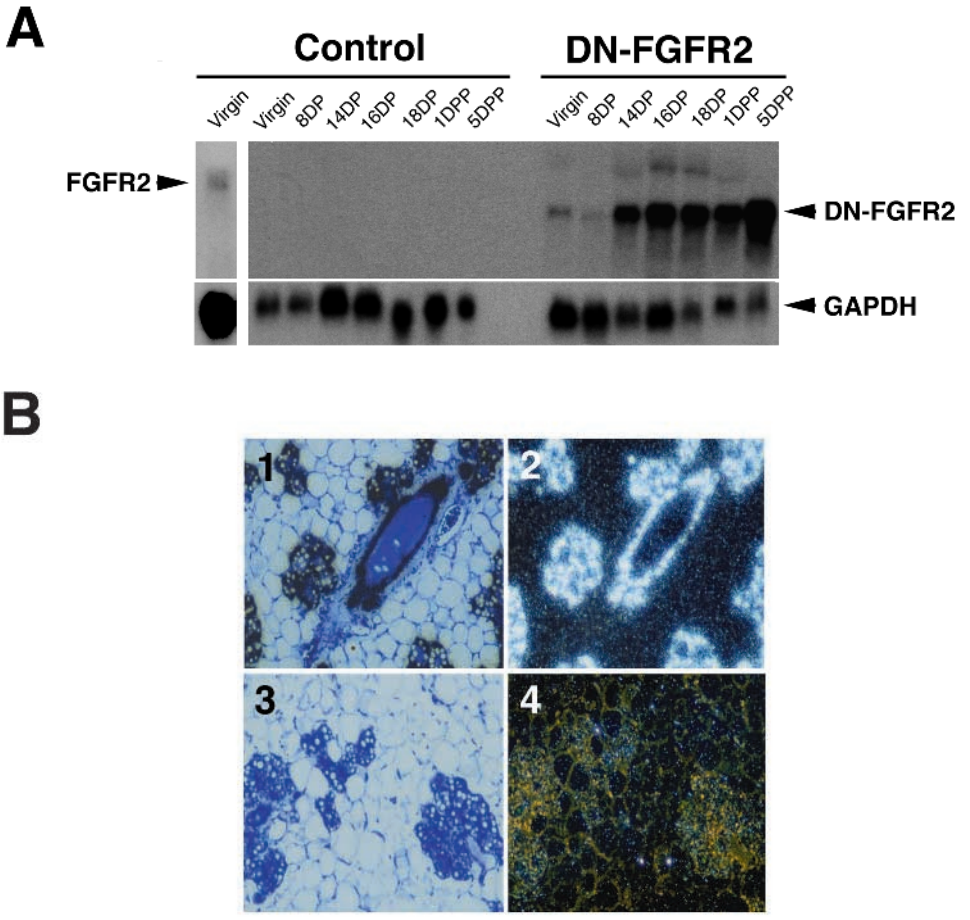

Fig. 5. Analysis of DN-FGFR2 RNA expression in mammary glands during pregnancy and lactation. (A) RNA samples (10 μg) from virgin, pregnant and lactating mammary glands from transgenic mouse line 18 and control glands were separated on denaturing 1% agarose gels, transferred to membranes and hybridized with a probe to *Fgfr2*. The endogenous receptor transcripts were not detected except at much longer exposure times (left panel). The transgene was found in all samples from DN-FGFR2 mice migrating at approximately 3.3 kb, as indicated. The same blot was rehybridized with GAPDH probe to assess RNA loading (lower panel). (B) In situ hybridization analysis of FGFR2 expression in DN-FGFR2 and control mammary glands. Formalin-fixed tissue sections of mid-pregnant mammary glands were hybridised with a ^{35}S-labelled *Fgfr2* antisense RNA, and counter-stained with Giemsa's stain. (1 and 3) Bright field and (2 and 4) the same views, in dark field illumination, of mid-pregnant mammary tissue from DN-FGFR2 (1 and 2) and control (3 and 4) mice, respectively.

Transgenic mice expressing DN-FGFR2

Two lines of transgenic mice (18 and 22) were selected for investigation on the basis of high DN-FGFR2 expression levels (data not shown). The females of both lines were found to have difficulty sustaining their young, as judged by the poor gain in weight of both transgenic and non-transgenic litter mates. To determine whether this phenotype correlated with expression of the transgene, RNA samples from virgin, pregnant and lactating female mice were compared with equivalent RNAs from control non-transgenic females (Fig. 5A). For both lines of transgenic mice, DN-FGFR2 was readily detected in mammary glands of virgin, pregnant and lactating mice. A particularly high level of the transgene was found during the latter half of pregnancy and at lactation, and this could in part reflect the accumulation of epithelial tissue that accompanies pregnancy. Endogenous FGFR2 RNA was not detected in samples from either control or transgenic mice at the exposure of the comparative northern blot shown in Fig. 5. However, endogenous FGFR2 can be detected as a band of approximately 4.5 kb in mammary glands at longer autoradiographic exposures times (Fig. 5A). In situ hybridisation using an FGFR2 probe demonstrated abundant transgene expression in large ducts and alveolar lobules of the mammary glands (Fig. 5B). No clear signal from endogenous FGFR2 was detected in glands of non-transgenic mice.

The morphological consequences of DN-FGFR2 expression on the mammary gland were investigated using whole mount preparations, and stained tissue sections. The gross appearance of the glands from virgin (not shown) and 8-day pregnant DN-FGFR2 transgenic (Fig. 6A) and control mice (Fig. 6E) were similar. However, by day 14 of pregnancy, a marked impairment of lobuloalveolar growth was observed in the glands from DN-FGFR2 transgenic females (Fig. 6B and F). This lack of lobular development was maintained through late pregnancy (Fig. 6C and G) and into lactation (Fig. 6D and H). Histological analysis of the antepartum transgenic mammary glands confirmed the retardation of development, but showed that the alveolar structure was retained despite the significant reduction in the number of units (compare B and C with H and I in Fig. 4). We estimated that by day 16 of pregnancy, less than 1/3 of the fat pad area is occupied by alveoli, compared to over half the area in control animals. However, despite their lack of growth, the transgenic glands were lactationally active as shown by alveolar dilation and the presence of basophilic secretion in the lumen (Fig. 4I). The low density of alveolar units appears to result in their greater dilation, presumably due to a less restricted environment in which to expand.

Milk protein analysis

Histological analysis of the postpartum mammary tissue from DN-FGFR2 mice suggested that the differentiation of the milk producing epithelium is relatively normal. As a biochemical assessment of this observation, we analysed post-partum mammary tissue from transgenic and control mice for the expression of milk proteins. Total RNA and protein extracts were prepared from normal and transgenic mammary glands

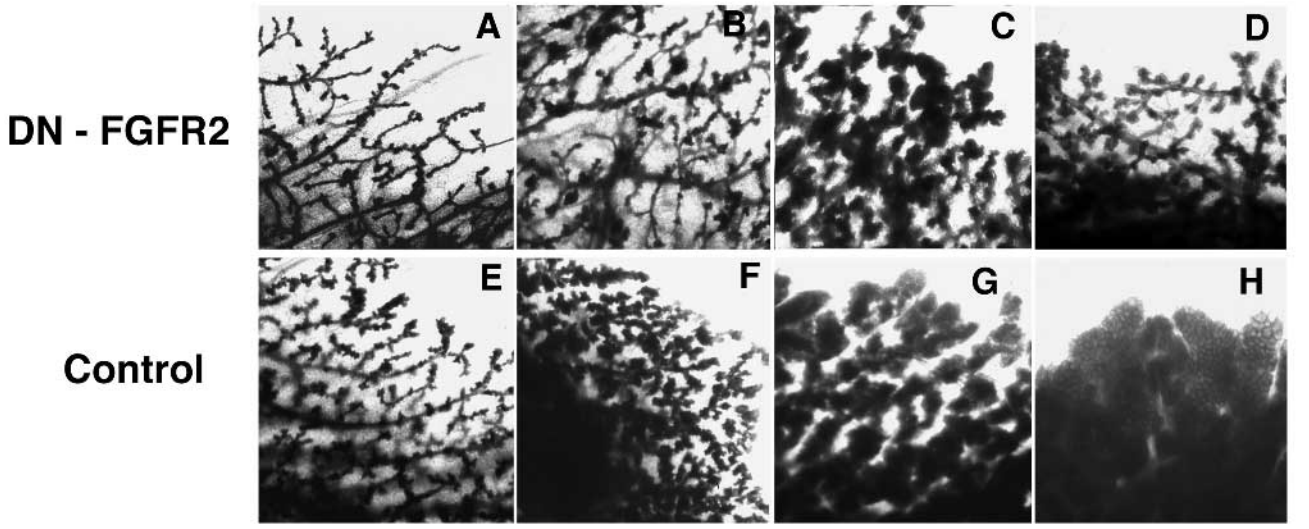

Fig. 6. Comparison of mammary glands from DN-FGFR2 transgenic (top panels) and control mice (bottom panels). Tissues were taken and prepared as whole mounts from 8-day pregnant (A and E), 14-day pregnant (B and F), 18-day pregnant mice (C and G) and 1-day post-partum mice (D and H). DN-FGFR2 are shown in A to D and controls in E to H. Magnification is approximately ×30.

of 16-day pregnant and 5-day postpartum mice. Northern blot analysis with a ^{32}P-labelled β-casein probe detected three major transcripts with estimated sizes of 1.4 kb, 1.1 kb and 0.9 kb (Fig. 7A). The 1.1 kb transcript is the predicted size for β-casein mRNA, while the two other transcripts are of a size compatible with α- and γ-casein mRNAs (Grusby et al., 1990; Yoshimura and Oka, 1989). The induction of these transcripts from a low abundance in the 16-day pregnant gland to the high levels seen in the lactating gland of control mice is the expected pattern for milk protein RNA expression. The transgenic post-partum mammary gland showed a weak signal compared to the wild-type gland indicating that the expected induction of casein mRNA was much lower in the transgenic line, which is consistent with reduced lobuloalveolar development. Similarly, when equivalent amounts of protein from the different mammary glands were separated by SDS-PAGE and immunoblotted with an antiserum to whole milk, proteins of the sizes expected for mouse caseins were detected in the samples from control mammary glands of postpartum mice but were at a substantially lower level in the equivalent transgenic glands (Fig. 7B).

DISCUSSION

Our current studies were undertaken to determine what role the FGFs might play in the normal development of the mammary gland. Previous work has demonstrated the presence of several FGFs and their receptors in breast tissue (Coleman-Krnacik and Rosen, 1994), and furthermore, the inappropriate expression of FGF3, FGF4, FGF7 and FGF8 have been shown to cause abnormal growth of the mammary epithelium demonstrating the responsiveness of this tissue to FGFs (MacArthur et al., 1995; Muller et al., 1990; Ornitz et al., 1992a; Peters 1991; Stamp et al., 1992; Kitsberg and Leder, 1996). However, establishing a role for FGF signalling is confounded by the presence of multiple FGFs that can signal through several different receptors (see Ornitz et al., 1996). Hence, the use of

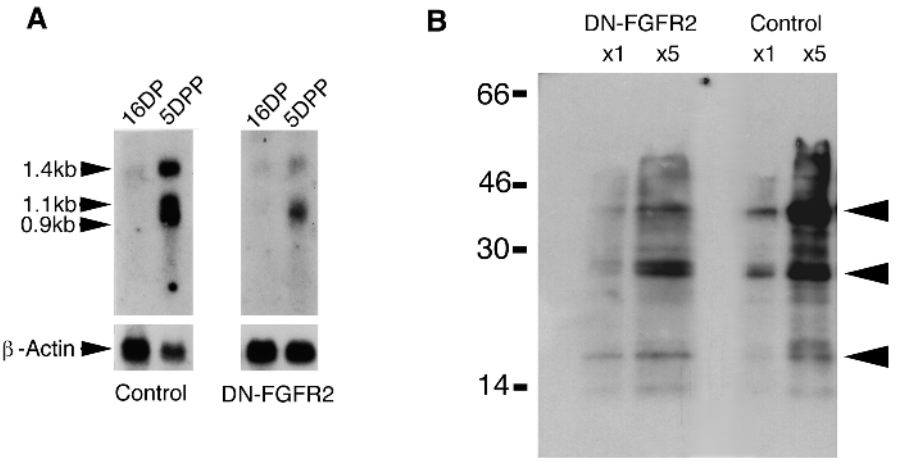

Fig. 7. Milk protein expression in mammary glands of ante- and post-partum mice. (A) A northern blot analysis of mammary tissue RNA (10 μg/track) from 16-day pregnant (16DP) and 5-day post-partum (5DPP) normal and transgenic mouse mammary glands hybridised with a probe to β-casein and β-actin. (B) An immunoblot analysis on protein extracts (5 μg) isolated from 5-day postpartum mammary tissue, and ×5 indicates a 5 fold higher loading of protein. A polyclonal rabbit antibody raised against whole milk protein and absorbed against virgin mammary tissue proteins was used as described. (Milk protein molecular masses: α-casein 35.6 kDa, β-casein 25.3, γ-casein 21.1 kDa, whey acidic protein 14.9 kDa.)

DN receptors can have advantages over germline deletions by affecting the signalling of different ligands that bind the same receptor, as well as different receptors which interact with the same ligand by sequestration of receptors through heterodimerisation (Kashles et al., 1991; Ueno et al., 1992). Here we have used kinase deleted forms of two FGF receptors, FGFR1(IIIc) and FGFR2(IIIb), to investigate FGF signalling during post-natal mammary gland development. For each transgene, two lines of mice were established where the DN receptor was expressed in the mammary epithelium in significant excess over the endogenous receptor level. An examination of many mammary glands, during pregnancy and lactation, from two different DN-FGFR1 transgenic mouse lines showed no significant differences to the equivalent control glands. This lack of a discernible phenotype is not likely to be due to a non-functional DN-receptor, since the transgene construct was shown to function efficiently in vitro. These results suggest that ligands such as FGF1, FGF2, FGF4, FGF5 or FGF6, which bind and activate FGFR1(IIIc), are unlikely to play an important role in the growth or differentiation of luminal epithelium during pregnancy or lactation. In contrast, the transgenic mouse lines expressing a kinase negative form of FGFR2(IIIb) resulted in a marked impairment of lobuloalveolar growth that was clearly apparent by mid-pregnancy. Histological and biochemical analysis indicated that the growth retardation persisted in the postpartum gland, although the lobules were able to differentiate and produce milk proteins. This finding provides clear evidence that normal lobuloalveolar development of the mammary gland depends on an FGF that can signal through FGFR2(IIIb). To date, three ligands are known to activate FGFR2(IIIb), FGF1, FGF3 and FGF7, and they are therefore candidates to function in the mammary gland. However, FGF1 is unlikely to be the ligand since it would be expected to give a similar phenotype with both transgenes as it can also bind and activate FGFR1(IIIc). FGF3 is excluded as it is not expressed in the mammary gland, although a role for signalling through FGFR2(IIIb) is consistent with the ability of FGF3 when aberrantly expressed to act as a potent oncogene in this organ (Peters, 1991; Stamp et al., 1992). This leaves FGF7 as the prime candidate for involvment in lobularalveolar development. This idea is consistent with its presence in the mammary gland (Coleman-Krnacik and Rosen, 1994, and unpublished results), and that it causes hyper-proliferation of the mammary parenchyma when injected systemically into rats or expressed as a transgene in this tissue (Kitsberg and Leder, 1996; Ulich et al., 1994). However, since mice deficient for *Fgf-7* have no reported abnormalities of the mammary gland, it cannot be the sole signalling ligand (Guo et al., 1996). Interestingly, *Fgf-7* null mice showed no lung or wound repair abnormalities, two other examples where signalling through FGFR2(IIIb) receptor has been established, and FGF7 implicated as the likely ligand (Guo et al., 1996; Peters et al., 1994; Werner et al., 1994). A possible alternative FGF for signalling through FGFR2(IIIb) is FGF10, a recently described member of the family which is highly homologous to FGF3 and FGF7 (Yamasaki et al., 1996). However, its receptor specificity and adult tissue distribution has yet to be reported. Nevertheless, we can conclude that there is a specific requirement for FGF signalling in normal lobuloalveolar development of the mammary gland during pregnancy.

We thank Gill Hutchinson for technical help and Vera Fantl, Laurence De Moerlooze and Gordon Peters for comments and critical reading of the manuscript. This work was supported in part by a grant from the Human Frontier Science Program.

REFERENCES

Amaya, E., Musci, T. J. and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. *Cell* **66**, 257-270.

Ball, R., Friis, R., Schonenberger, C., Doppler, W. and Groner, B. (1988). Prolactin regulation of b-casein gene expression and of a cytosolic 120 kd protein in a cloned mouse mammary epithelial cell line. *EMBO J.* **7**, 2089-2095.

Banerjee, M., Wood, B., Lin, F. and Crump, L. (1976). Organ culture of whole mammary gland of the mouse. In *Tissue Culture Association Manual*, vol. 2 (ed. Sandford, K. K.), pp. 457-462. Gaithersberg, MD: TCA.

Basilico, C. and Moscatelli, D. (1992). The FGF family of growth-factors and oncogenes. *Advan. Cancer Res.* **59**, 115-165.

Buhler, T., Dale, T., Kieback, C., Humphreys, R. and Rosen, J. (1993). Localization and quantification of Wnt-2 gene expression in mouse mammary development. *Dev. Biol.* **155**, 87-96.

Burgess, W. H. and Maciag, T. (1989). The heparin binding (fibroblast) growth factor family proteins. *Annu. Rev. Biochem.* **58**, 575-606.

Chellaiah, A., McEwen, D., Werner, S., Xu, J. and Ornitz, D. (1994). Fibroblast growth factor receptor (FGFR) 3. *J. Biol. Chem.* **269**, 11620-11627.

Cheon, H. G., Larochelle, W. J., Bottaro, D. P., Burgess, W. H. and Aaronson, S. A. (1994). High-affinity binding-sites for related fibroblast growth-factor ligands reside within different receptor immunoglobulin-like domains. *Proc. Nat. Acad. Sci. USA* **91**, 989-993.

Coleman-Krnacik, S. and Rosen, J. M. (1994). Differential temporal and spatial gene-expression of fibroblast growth-factor family members during mouse mammary-gland development. *Mol. Endocrinol.* **8**, 218-229.

Daniel, C. and Silberstein, G. (1987). Postnatal development of the rodent mammary gland. *The Mammary Gland: Regulation, and Function*. (ed. M. C. Neville and C. W. Daniel), pp. 3-36. New York: Plenum Press.

Gavin, B. and McMahon, A. (1992). Differential regulation of the Wnt gene family during pregnancy and lactation suggests a role in postnatal development of the mammary gland. *Mol. Cell. Biol.* **12**, 2418-2423.

Grusby, M., Mitchell, S., Nabavi, N. and Glimcher, L. (1990). Casein expression in cytotoxic T lymphocytes. *Proc. Nat. Acad. Sci. USA* **87**, 6897-6901.

Guo, L., Degenstein, L. and Fuchs, E. (1996). Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.* **10**, 165-175.

Hogan, B., Constantini, F. and Lacy, E. (1986). *Manipulating The Mouse Embryo: a Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Jaye, M., Schlessinger, J. and Dionne, C. (1992). Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. *Biochem. Biophys. Acta.* **1135**, 185-199.

Jhappan, C., Geiser, A., Kordon, E., Bagheri, D., Hennighausen, L., Roberts, A., Smith, G. and Merlino, G. (1993). Targeting expression of a transforming growth factor β1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. *EMBO J.* **12**, 1835-1845.

Johnson, D. and Williams, L. (1993). Structural and functional diversity in the FGF receptor multigene family. *Advan. Cancer Res.* **60**, 1-41.

Kashles, O., Yarden, Y., Fisher, R., Ullrich, A. and Schlessinger, J. (1991). A dominant negative mutation suppresses the function of normal epidermal growth factor receptors by heterodimerization. *Mol. Cell. Biol.* **11**, 1454-1463.

Kiefer, P., Mathieu, M., Close, M., Peters, G. and Dickson, C. (1993). FGF3 from Xenopus-laevis. *EMBO J.* **12**, 4159-4168.

Kitsberg, D. and Leder, P. (1996). Keratinocyte growth-factor induces mammary and prostatic hyperplasia and mammary adenocarcinoma in transgenic mice. *Oncogene* **13**, 2507-2515.

MacArthur, C. A., Shankar, D. B. and Shackleford, G. M. (1995). FGF-8, activated by proviral insertion, cooperates with the wnt-1 transgene in murine mammary tumorigenesis. *J. Virol.* **69**, 2501-2507.

Mathieu, M., Kiefer, P., Mason, I. and Dickson, C. (1995). Fibroblast growth-factor (FGF)-3 from Xenopus-laevis (xfgf3) binds with high-affinity to FGF receptor-2. *J. Biol. Chem.* **270**, 6779-6787.

Morgenstern, J. P. and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucl. Acids Res.* **18**, 3587-3596.

Muller, W. J., Lee, F. S., Dickson, C., Peters, G., Pattengale, P. and Leder, P. (1990). The int-2 gene product acts as an epithelial growth factor in transgenic mice. *EMBO J.* **9**, 907-913.

Ornitz, D. M., Cardiff, R. D., Kuo, A. and Leder, P. (1992a). Int-2, an autocrine and/or ultra-short-range effector in transgenic mammary tissue transplants. *J. Nat. Cancer Inst.* **84**, 887-892.

Ornitz, D. M. and Leder, P. (1992). Ligand specificity and heparin dependence of fibroblast growth-factor receptor-1 and receptor-3. *J. Biol. Chem.* **267**, 16305-16311.

Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E. and Leder, P. (1992b). Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol. Cell. Biol.* **12**, 240-247.

Ornitz, D. M., Xu, J. S., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G. X. and Goldfarb, M. (1996). Receptor specificity of the fibroblast growth-factor family. *J. Biol. Chem.* **271**, 15292-15297.

Peters, G. (1991). Inappropriate expression of growth factor genes in tumors induced by mouse mammary tumor virus. *Semin. Virol.* **2**, 319-328.

Peters, K., Werner, S., Liao, X., Wert, S., Whitsett, J. and Williams, L. (1994). Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. *EMBO J.* **13**, 3296-3301.

Pierce, D. Jr, Johnson, M., Matsui, Y., Robinson, S., Gold, L., Purchio, A., Daniel, C., Hogan, B. and Moses, H. (1993). Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-β1. *Genes Dev.* **7**, 2308-2317.

Ruan, W., Newman, C. and Kleinberg, D. (1992). Intact and amino-terminally shortened forms of insulin-like growth factor I induce mammary gland differentiation and development. *Proc. Nat. Acad. Sci. USA* **89**, 10872-10876.

Senior, P., Critchley, D., Beck, F., Walker, R. and Varley, J. (1988). The localisation of laminin mRNA and protein in the postimplantation embryo and placenta of the mouse: an in situ hybridisation and immunocytochemical study. *Development* **104**, 431-446.

Snedeker, S., Brown, C. and DiAugustine, R. (1991). Expression and functional properties of transforming growth factor α and epidermal growth factor during mouse mammary gland ductal morphogenesis. *Proc. Nat. Acad. Sci. USA* **88**, 276-280.

Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladbury, J. E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J. and Lax, I. (1994). Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell-proliferation. *Cell* **79**, 1015-1024.

Stamp, G., Fantl, V., Poulsom, R., Jamieson, S., Smith, R., Peters, G. and Dickson, C. (1992). Nonuniform expression of a mouse mammary tumor virus-driven *int-2*/*Fgf-3* transgene in pregnancy-responsive breast tumors. *Cell Growth Differ.* **3**, 929-938.

Tso, J., Sun, X.-H., Kao, T.-h., Reece, K. and Wu, R. (1985). Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucl. Acids Res.* **13**, 2485-2501.

Ueno, H., Gunn, M., Dell, K., Tseng, A. and Williams, L. (1992). A truncated form of fibroblast growth-factor receptor-1 inhibits signal transduction by multiple types of fibroblast growth-factor receptor. *J. Biol. Chem.* **267**, 1470-1476.

Ulich, T., Yi, E., Cardiff, R., Yin, S., Bikhazi, N., Biltz, R., Morris, C. and Pierce, G. (1994). Keratinocyte growth factor is a growth factor for mammary epithelium in vivo. *Am. J. Path.* **144**, 862-868.

Werner, S., Weinberg, W., Liao, X., Peters, K., Blessing, M., Yuspa, S., Weiner, R. and Williams, L. (1993). Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation. *EMBO J.* **12**, 2635-2643.

Werner, S., Smola, H., Liao, X., Longaker, M., Krieg, T., Hofschneider, P. and Williams L. (1994). The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. *Science* **266**, 819-822.

Wilkie, A. O. M., Morrisskay, G. M., Jones, E. Y. and Heath, J. K. (1995). Functions of fibroblast growth-factors and their receptors. *Curr Biol.* **5**, 500-507.

Wright, N. A., Poulsom, R., Stamp, G. W. H., Hall, P. A., Jeffery, R. E., Longcroft, J. M., Rio, M., Tomasetto, C. and Chambon, P. (1990). Epidermal growth factor (EGF/URO) induces expression of regulatory peptides in damaged human gastrointestinal tissues. *J. Pathol.* **162**, 279-284.

Yamasaki, M., Miyake, A., Tagashira, S. and Itoh, N. (1996). Structure and expression of the rat mRNA encoding a novel member of the fibroblast growth factor family. *J. Biol. Chem.* **271**, 15918-15921.

Yanmin, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, K., Birchmeier, C. and Birchmeier, W. (1995). Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. *J. Cell Biol.* **131**, 215-226.

Yoshimura, M. and Oka, T. (1989). Isolation and structural analysis of the mouse beta-casein gene. *Gene* **78**, 267-275.

Zimmer, Y., Givol, D. and Yayon, A. (1993). Multiple structural elements determine ligand-binding of fibroblast growth-factor receptors - evidence that both ig domain-2 and domain-3 define receptor specificity. *J. Biol. Chem.* **268**, 7899-7903.

(Received 24 February 1997 - Accepted 25 March 1997)