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Retroviral infection of the *FGF2* gene into MCF-7 cells induces branching morphogenesis, retards cell growth and suppresses tumorigenicity in nude mice

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Abstract

FGF2 (basic fibroblast growth factor) is a multifunctional growth factor and exhibits diverse function in different cell types. In breast, loss of FGF2 expression is associated with malignant progression. In order to understand the role of FGF2 in maintenance of normal breast structures and control of cell growth, we restored *FGF2* expression in the breast cancer cell line MCF-7. The FGF2 retrovirally infected MCF-7 cells (MCF-7.F2.5) not only expressed FGF2 in cytoplasm and nuclei, but also released FGF2 into culture medium both on plastic and in Matrigel conditions. The MCF-7.F2.5 cells formed branches in Matrigel and this effect was abolished by the addition of a neutralising anti-FGF2 antibody or function blocking antibodies to α2, α3 and β1 integrins. Furthermore, MCF-7.F2.5 cells lost their ability for anchorage-independent growth in soft agar. When MCF-7 and MCF-7.F2.5 cells were injected into nude mice, there was a 1.6- to 3.2-fold reduction of tumour volume with MCF-7.F2.5 cells in comparison with the parental MCF-7 cells. MCF-7.F2.5 cells also demonstrated a reduction in oestrogen receptor-α (ERα) both *in vitro* and *in vivo*. Our results suggest that introduction of the *FGF2* gene into MCF-7 cells altered the malignant tumour cells towards a more benign phenotype *in vitro* and *in vivo*.

Keywords: Basic fibroblast growth factor; Breast cancer; Morphogenesis; Tumorigenicity; Oestrogen receptor

1. Introduction

FGF2 is the prototype member of a large family of structurally related, heparin binding, polypeptide growth factors and is found in virtually all tissues studied, both normal and malignant. Classically it is known as an angiogenic factor, stimulating the proliferation and invasion of the endothelial cells required for capillary formation as well as being a potent mitogen for fibroblast cells [1]. However, FGF2 is now known to be pleiotropic, having a variety of functions depending on its location.

Like FGF1 and FGF9, FGF2 is synthesised without the N-terminal hydrophobic signal sequence and is therefore considered not to be secreted via conventional means. The presence of plasma membrane receptors [2,3], the inhibitory effects of neutralising antibodies [4,5], its location in basement membranes *in vivo* [6,7] and its presence in cell culture conditioned medium *in vitro* [8] have indicated an extracellular mechanism of action for FGF2. It is thought that release may occur through leakage from damaged cells or from viable cells via a novel process which is not dependent on the endoplasmic reticulum-Golgi pathway [9].

FGF2 has been studied extensively as having a potential role as an epithelial mitogen in a variety of cancers, including those of the oesophagus [10], pancreas [11], colon [8], bladder [12], ovary [13] and brain [14]. However, lower levels of FGF2 mRNA have been found in malignant human breast biopsies than in normal and benign breast tissues [3] and this has since been confirmed at the protein level [15,16]. In addition, higher levels of FGF2 are associated with improved overall and disease-free survival [16]. Immunohistochemistry

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of human breast sections [7] and analysis of mRNA and protein extracted from purified normal human breast cell populations [17] has identified the myoepithelial cell as the main source of FGF2 in the breast and this cell type is lost in the progression to neoplasia, suggesting a role of myoepithelial-derived FGF2 in control of epithelial cell growth.

With respect to breast cancer epithelial cell lines, there are conflicting reports on the effect of FGF2 on their growth. In the absence of serum, FGF2 has been shown to have variable stimulatory effects on the proliferation of MCF-7 and T47D cells [18,19] with Stewart and associates [20] reporting that oestradiol is required for FGF2 growth stimulation of MCF-7 cells. Recently, however, FGF2 has been shown to be inhibitory for MCF-7 cell growth in the presence of serum, despite activating the MAP kinases, ERK1 and ERK2 (extracellular signal-regulated kinases), which are generally thought to signal growth stimulatory responses [21,22]. Similarly, FGF2 has been demonstrated to be inhibitory to the oestrogen receptor negative cell line MDA-MB-134 which overexpresses the FGF receptor, FGFR1 [23] and to have no effect on the proliferation of the MDA-MB-231 cells [19]. The latter may be due to there already being an autocrine stimulation of growth by the FGF1 and FGF2 produced by these cells [5]. Therefore, identification of the role of FGF2 in breast cancer promotion or suppression would not only lead to a better understanding of the natural history of breast cancer, but also to development of a new modality of intervention of this disease.

The effect of FGF2 on cell differentiation and morphogenesis has not been broadly studied. It has been shown to induce differentiation of neural cells *in vitro* and neural outgrowth *in vivo* [24], as well as having a role in embryogenesis and mesoderm induction [25]. Our previous study demonstrated that the formation of bilayer lobuloalveolar structures in a human breast *in vitro* model system is dependent on the presence of both epithelial and myoepithelial cells within a basement membrane matrix (Matrigel) and is enhanced in the presence of FGF2 [26]. In addition, Bergstraesser and associates [27] have shown that neutralising antibodies to FGF2 inhibit duct formation by mixed normal human breast cells on top of Matrigel. However, these studies only showed a combinative effect of myoepithelial cells and FGF2 on mammary morphogenesis. The direct role of FGF2 on the morphogenesis of mammary luminal epithelial cells is still unclear.

Oestrogens are important for reproductive tract and mammary gland growth and development. There is a strong correlation between the action of oestrogens (particularly 17β-oestrdiol) and carcinogenesis in breast. The biological function of oestrogen hormones are mediated through oestrogen receptor alpha (ERα), a member of the superfamily of transcriptional factor nuclear receptors. There are several lines of evidence that oestrogens/ERα signalling can regulate growth factor expression and secretion [28]. Similarly, growth factors and hormones can also modulate oestrogen receptor expression and function [29].

In this study, we provide evidence that FGF2, when overexpressed in MCF-7 cells, exerts multiple effects, which may all contribute to a tumour suppressor role in breast cancer. We find that FGF2, in addition to slowing the rate of anchorage-dependent growth, almost completely inhibited anchorage-independent growth, induces branching morphogenesis in a basement membrane matrix which is α2β1 α3β1 integrin dependent, and also downregulates oestrogen receptor expression. Taken together these effects could result in a substantial inhibition of tumour growth *in vivo*.

2. Materials and methods

2.1. Cell culture

MCF-7 cells (ATCC, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, Dorset, UK) supplemented with 0.4 mM L-glutamine, 20 U/ml penicillin, 20 μg/ml streptomycin (Sigma) and 10% fetal calf serum (FCS, Gibco BRL Life Technologies, Renfrewshire, UK) at 37°C in 5% CO_2 at 100% humidity.

2.2. Retroviral infection

The retroviral producer cell line PA317-pbabe FGF2*neo* was a gift from N. Lemoine (ICRF, London, UK). Cells were cultured in DMEM and 10% FCS supplemented with 1 mg/ml G418 (neomycin analogue, Gibco BRL Life Technologies) for 3 days until confluent. This medium was then replaced with fresh medium without G418 overnight. The viral supernatants were harvested and then incubated with MCF-7 cells in the presence of 8 μg/ml polybrene (Sigma) as carrier. After overnight incubation, the viral supernatants were replaced with fresh culture medium and cells incubated for a further 48 h. The cells were trypsinised and diluted in medium containing 1.2 mg/ml of G418 at 30 cells/ml to select for neomycin-resistant FGF2-transduced cells. 100 μl of cell suspension was added into each well of 96-well plates. The wells which contained only one cell were selected. These single cells were propagated to cell lines (subclones).

2.3. Cell growth in Matrigel

2×10^4 cells were quickly mixed with ice-cold Matrigel (Stratech Scientific Ltd, Bedfordshire, UK) at a protein concentration of 10–12 mg/ml. This cell/basement mem-

brane matrix mixture was then aliquotted (300 μl/well) into four-well glass chamber slides (Nunc, Gibco BRL Life Technologies) and warmed for 20 min to allow the gel to set. 500 μl of culture medium was added on top of the gel in each well. Progress was monitored by phase contrast microscopy using a Zeiss Axiovert 10 inverted microscope. Photographs were taken every 3 days for 3 weeks. In the antibody blocking experiments, serial concentrations (12.5, 25, 50 and 100 μg/ml) of an anti-FGF2 monoclonal antibody, (R & D Systems, Oxon, UK) and anti-integrin β1, α2, α2, α3, and α6 subunit antibodies (Chemicon, Harrow, UK) at 10–50 μg/ml were added to the media on top of the Matrigel every 3 days for 3 weeks. The experiments were performed in triplicates.

2.4. Cell growth assay

Growth kinetics were assessed using a SRB (Sulphorhodamine B) assay which is based on staining basic amino acids in cells. Cells were harvested by means of a trypsin–EDTA mixture (1.25% trypsin and 0.02% EDTA), resuspended in DMEM/10% FCS and plated at 2×10^3 cells/well in six replicates in 96-well plates. At day 1 (24 h), day 3, and day 6, different plates were harvested by incubating cells with ice-cold 40% trichloroacetic acid at 4°C for 1 h. After washing in tap water, cells were stained with 0.4% SRB for 30 min. The plates were then washed with 1% acetic acid five times to remove the remaining dye and left to air-dry overnight. On the day of reading the plates, 100 μl of 10 mM Tris base was added to each well. Plates were vibrated for 15 min prior to reading their optical density in a scanning spectrophotometer (Labsystems, Hants, UK) at a wavelength of 492 nm.

2.5. Anchorage-independent growth in soft agar

The basal gel was prepared by mixing 1.6% autoclaved agar (Sigma) with an equal volume of 2×DMEM/20% FCS and then plating on to six-well plates (Nunc). 1×10^3 trypsinised cells were resuspended in 0.6% autoclaved agar and mixed with an equal volume of 2×DMEM/20% FCS before they were overlaid on the basal gel. Triplicate wells were set up for each cell line. After 14 days culture at 37°C in 10% CO_2, cell clusters containing more than 30 cells were scored as colonies. Colonies were counted by phase contrast microscopy.

2.6. Immunostaining

Cells cultured in four-well glass chamber slides were fixed in 3.7% PBS buffered formalin and permeabilised in ice-cold 50% acetone followed by 100% acetone for 5 min each. Cells were then washed in PBS, and blocked for endogenous biotin, following the protocol included with the biotin blocking kit (Vectastain). After three washes, cells were pre-incubated with blocking buffer (PBS containing 10% goat serum, 5% bovine serum albumin (BSA)) for 30 min. This buffer was removed and replaced with a monoclonal antibody to FGF2 (Oncogene Research Products, Cambridge, UK) at a concentration of 0.5 μg/ml, diluted in blocking buffer. Mouse IgG at the same concentration was used in control wells. After incubation at 4°C overnight, cells were washed three times in PBS and then incubated in biotinylated horse anti-mouse IgG for 1 h. After three washes, the cells were incubated with an avidin–biotin horseradish peroxidase (HRP) complex (Vectastain) for 30 min and staining visualised using 0.05% 3,3-diminobenzidine/0.01% hydrogen peroxide. Nuclei were counterstained with Gill's haematoxylin.

For oestrogen receptor alpha (ERα) staining, all procedures were the same as described above except that Tris buffer (pH 7.6) was used for washing and an anti-ERα monoclonal antibody as the primary antibody (Dako-ER, 1D5, Dako, Bucks, UK) at 1:100 dilution. Paraffin sections (8 μm) of tumours from nude mice, after dewaxing, were treated in a pressure cooker for 2 min in citrate buffer (pH 6.0) prior to immunohistochemical staining for ERα.

For endothelial cell marker CD31 staining, an indirect immunofluorescence method was performed. Harvested tumour tissues were snap frozen in liquid nitrogen and cut into 7 μm thick sections. Samples were allowed to air dry and subsequently fixed in cold acetone for 10 min. After two washes, samples were blocked with PBS containing 1% BSA and 5% rabbit serum for 1 h at room temperature. The blocking buffer was removed and replaced by rat antimouse CD31 antibody (Serotec, Oxford, UK) at 1 μg/ml dilution. After incubation at room temperature for 30 min, samples were washed three times with PBS and subsequently incubated with rabbit antirat fluorescein isothiocyanate (FITC) conjugated antibody (Sigma) for 30 min at room temperature. After further three washes with PBS, slides were mounted with glycerol/PBS and visualised with Zeiss florescence microscope. Estimation of microvessel density was performed by counting CD31 positive blood vessels per microscopic field for five randomly selected fields on each slide at 200× magnification.

2.7. FACS analysis

Cells were trypsinised and washed twice with PBS containing 1% FCS (4°C). Approximately 2×10^6 cells in 100 μl PBS/1% FCS were incubated with mouse monoclonal antibodies to β1 (B3B11, 1 μg/ml), α2 (P1E6, 1 μg/ml), α3 (P1B5, 1:100) and α6 (4F10, 1 μg/ml) integrins (Chemicon) for 60 min on ice. After two washes with the same medium, cells were incubated with an antimouse FITC conjugated second antibody

(Sigma, 1:100) for 30 min on ice. Cells were then washed with PBS/1% FCS three times and analysed using FACScan (Epics XL, Coulter, Hialeah, FL, USA).

2.8. Western blot analysis

Cells cultured in monolayer were trypsinised and washed with ice-cold PBS. Following centrifugation at 1300 rpm for 10 min, the cell pellets were solubilised in standard sample buffer. Forty micrograms of protein was loaded on to each lane of a 15% polyacrylamide gel. Separated proteins were electro-transferred on to nitrocellulose membranes. Non-specific sites were blocked with 5% non-fat milk power in tri-buffered saline (TBS) supplemented with 0.05% tween 20 for 30 min. The membrane was then incubated with 0.5 μg/ml of an anti-FGF2 monoclonal antibody (Oncogene Research Products), followed by a further 1 h incubation in a rabbit antimouse second antibody conjugated to HRP. After four washes, bands were visualised using the ECL method (Amersham Life Science Ltd, Essex, UK).

2.9. Detection of released FGF2 by ELISA

Conditioned media from cells cultured in monolayer and Matrigel were collected to measure the release of FGF2. For cells cultured in Matrigel, 72 h conditioned media were collected on day 10, and for monolayer cultures, media were collected at 80% confluence. All conditioned media were centrifuged at 1000 rpm for 10 min to remove adherent cells and the supernatants analysed using a Quantikine human FGF2 ELISA kit according to the manufacturers instructions (R & D Systems). Briefly, 50 μl of blocking solution was added to 96-well plates already coated by R & D with an antibody to FGF2. Two hundred microlitres of culture supernatants, neat or at a 1:1 dilution in triplicates were added to each well and incubated for 2 h at room temperature. After washing with buffer three times, 200 μl of a second antibody to FGF2 conjugated to HRP was added to each well and incubated at room temperature for another 2 h. Following further washes, 200 μl of substrate solution was added to each well and incubated for 20 min. After the addition of 50 μl of stop solution, the absorbance was read immediately using a scanning spectrophotometer (Labsystems) at 450 nm.

2.10. Northern blot analysis

Total RNA was extracted by using the guanidium isothiocyanate method. Northern blot analysis of RNA was carried out by the glyoxylation method. Glyoxylated RNAs were resolved by electrophoresis on 1.5% agarose gels run in re-circulating 10 mM sodium phosphate buffer. Resolved RNA gels were blotted by capillary action on to Hybond N hybridisation filter (Amersham Life Science Ltd) in autoclaved 10×standard saline citrate (SSC). Glyoxal was removed from blotted RNA by baking at 80°C for 1.5 h. Tracks containing resolved RNA size markers were cut away from the filter blot and visualised by fixing in 1 M acetic acid for 10 min, followed by staining in 0.4 M sodium acetate, 0.2% (w/v) methylene blue. Background Methylene Blue stain was removed by repeated washing in autoclaved, distilled water. Blotted RNA was hybridised to a human oestrogen receptor alpha (ERα) cDNA fragment, corresponding to the first 1.4 Kb of coding sequence of the clone HEG0 [30]. Post-hybridisation, filters were sequentially washed under increasingly stringent conditions of 5×SSC, 0.1% sodium dodecylsulphate (SDS) for 30 min, 2×SSC, 0.1% SDS for 30 min with the final wash in 0.5×SSC, 0.1% SDS at 65°C for 20 min. Autoradiography was performed at −70°C using Kodak XAR-2 film, with an intensifying screen. Filters were rehybridised with a ribosomal RNA probe, in order to correct for loading differences between samples analysed.

2.11. Tumorigenicity

Forty-six-week old ovariectomised nude mice were randomly divided into two groups. 1×10^7 trypsinised MCF-7 or MCF-7.F2.5 cells in 0.2 ml of growth medium were subcutanously injected into one flank of each mouse. Nude mice also received 1.5 mg 60 day release 17β-oestradiol pellets (Innovative Research of America, FL, USA) which were subcutaneously implanted between the scapulae 6 days before injection of cells. Tumour size was measured each week. Nude mice were sacrificed at the end of week 4 or after one diameter of the tumour reached 15 mm. Tumour volume was calculated by methods described elsewhere [31]. The Student's *t*-test was used to determine whether differences in tumour volume between MCF-7 and MCF-7.F2.5 cell groups were statistically significant. Tumours were embedded in paraffin, sectioned and stained with haematoxylin and eosin, as well as for oestrogen receptor alpha.

3. Results

3.1. Retroviral infection of FGF2 gene into breast carcinoma cells

The retroviral producer cell line pbabeFGF2*neo* had been previously subcloned (N Lemoine, ICRF, London). The highest viral titer clone was used for this study. MCF-7 cells were incubated with supernatants from pbabeFGF2*neo* cells overnight. After culture in normal growth medium for 48 h, the cells were split 1 in 10 and incubated with growth medium containing G418 (1 mg/ml) for a further 2 weeks. A 10–15% infection

take-rate was achieved in MCF-7 cells as seen by immunocytochemical staining for FGF2 (Fig. 1). These cells were then subcloned by a limited dilution technique. Clone 5 of the MCF-7 cells demonstrated 100% FGF2 positive cells and was designated MCF-7.F2.5 (Fig. 1). Clones 1 and 3 of the MCF-7 cells were both negative for FGF2 expression and were designated MCF-7.F2.1 and MCF-7.F2.3. These cells were used as negative controls. FGF2 was localised in both the nucleus and cytoplasm of the MCF-7 transfectants. The synthesis of all molecular weight isoforms of FGF2 were confirmed by western blotting of MCF-7.F2.5 cells (Fig. 1).

3.2. Release of FGF2

In order to examine secreted FGF2, cell culture supernatants taken from monolayer and Matrigel cultures were measured by ELISA. FGF2 was detected in the conditioned medium from the FGF2 positive clone MCF-7.F2.5 when cells were cultured on plastic and embedded in Matrigel, but not from the negative controls MCF-7.F2.1 and MCF-7.F2.3 and the parental MCF-7 cells. The production of FGF2 was 500-fold higher from cells embedded inside Matrigel in comparison with cells cultured on plastic (Fig. 2), suggesting that the extracellular matrix (laminin and collagen) may stimulate FGF2 production or release from MCF-7.F2.5 cells.

3.3. Morphogenesis in Matrigel

There were no morphological differences between MCF-7 and their FGF2 infected counterparts in monolayer culture. However, when these cells were embedded in Matrigel, a reconstituted basement membrane matrix

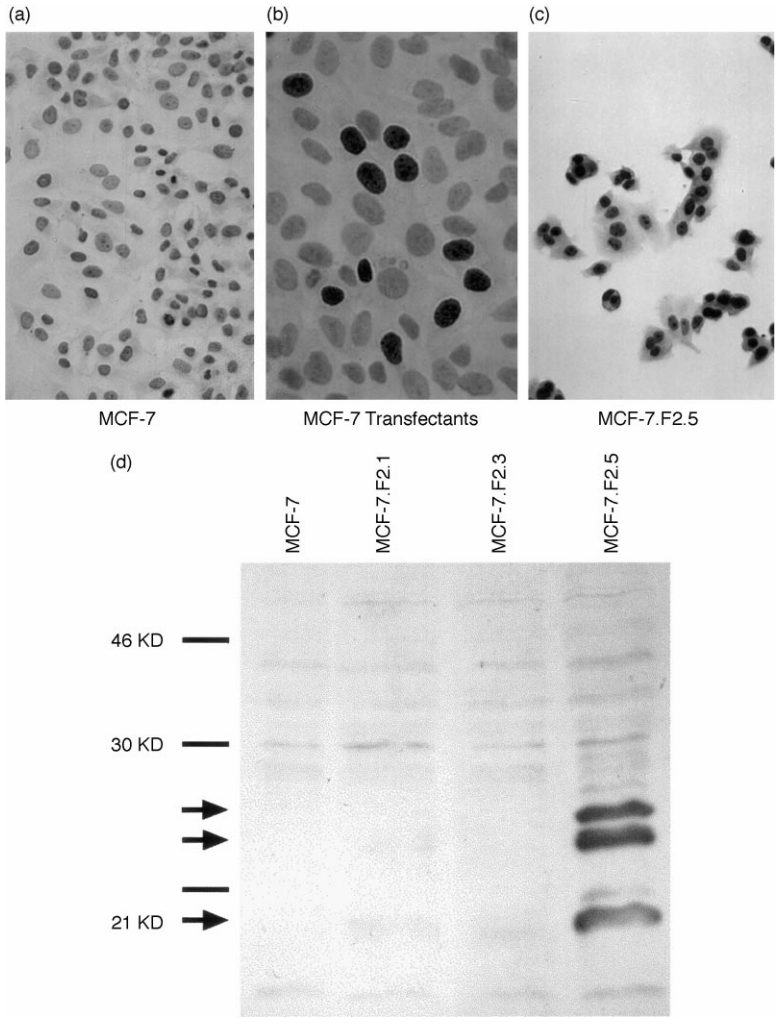

Fig. 1. Expression of FGF2 in MCF-7 cells: immunoperoxidase staining for FGF2 in (a) MCF-7 parental cells; (b) FGF2 transfectants and (c) the subclone MCF-7.F2.5. Magnification ×200. Uncloned cells show mainly nuclear staining (b), cloned cells (c) have cytoplasmic and nuclear staining; (d) western blot analysis of cell lysates of MCF-7 parental and retrovirally infected subclones; arrows indicate different molecular weight bands of FGF2 infected subclones. All four molecular isoforms of FGF2 (18, 22, 22.5 and 26 kDa) were expressed.

which contains laminin and collagen IV, MCF-7.F2.5 cells started to form branching structures at week 1 and these branches gradually extended with time (Fig. 3). A colony containing more than five branches was considered to be a branching structure. About 70% of MCF-7.F2.5 cell clusters formed branching structures. The negative controls MCF-7.F2.1 and MCF-7.F2.3 and parental MCF-7 cells only formed spheroids (Fig. 3). H & E staining of paraffin sections through the branches showed some cellular organisation with the cells lined up in two layer cords with no lumen formation.

Since FGF2 was secreted by MCF-7.F2.5 cells, serial concentrations of a neutralising anti-FGF2 antibody were applied to Matrigel cultures at day 0 and then every 3 days up to 3 weeks. A dose-dependent inhibition of branching morphogenesis was seen with the anti-FGF2 antibody between 25 and 100 μg/ml (Fig. 4). This data suggests that MCF-7.F2.5 cells form branching structures via an autocrine loop providing that a sufficient amount of FGF2 is available. Moreover, addition of recombinant human FGF-2 (20 ng/ml) to MCF-7 cells in Matrigel also resulted in similar structures being formed to those seen with MCF-7.F2.5 cells (data not shown). These results confirm that FGF2 is a morphogen for MCF-7 cells.

3.4. Effect of anti-integrin antibodies on MCF-7/FGF2 transfectants in Matrigel

Because branching structures were only formed when cells were cultured in Matrigel, which contains laminin and collagen IV, and not on a plastic surface, this suggests that an interaction between the extracellular matrix molecules and their receptor integrins may play a critical role in MCF-7.F2.5 branching morphogenesis. Expression of the integrin subunits β1, α2 α3 and α6 in MCF-7 and FGF2 infected counterparts was examined by FACS analysis. All MCF-7, MCF-7.F2.1, MCF-7.F2.3 and MCF-7.F2.5 cells expressed β1, α2 and α3 integrin subunits, but there was no difference in the level of fluorescence intensity between any of these cells (Fig. 5). All cell lines showed very low fluorescence intensity for α6 integrin with a 10-fold reduction in comparison with β1, α2 and α3 integrin subunits (Fig. 5).

Anti-integrin antibodies were applied to the MCF-7.F2.5 cells cultured in Matrigel (Fig. 6). It was found that the anti-β1 integrin antibody at 25 μg/ml inhibited 76% of branching and at 50 μg/ml completely inhibited branch formation. Cells formed round and tight spheroids. MCF-7.F2.5 cells incubated with antibodies to α2 and α3 integrins at 10 μg/ml also failed to form branching structures. Interestingly, in the presence of

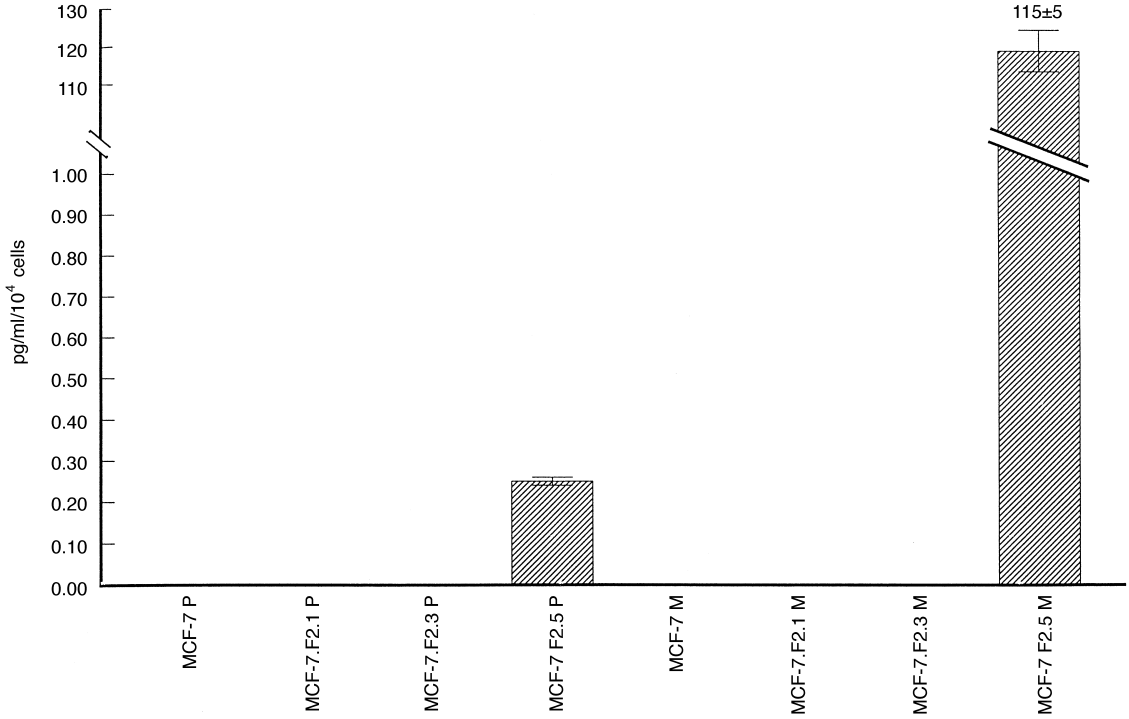

Fig. 2. The production of FGF2 from MCF-7 and their FGF2 infected counterparts was measured by ELISA. The 72 h conditioned media from cells cultured on plastic (P), in Matrigel (M), were collected on day 10. The ELISA was performed according to manufacturer's instructions. Triplicate wells were used for each sample. The mean OD value for each sample was converted to the equivalent amount of human FGF2 using a standard curve (not shown) and then corrected for cell number. The values represent the production of FGF2 in pg per ml per 10 000 cells. Standard errors are shown by the bars.

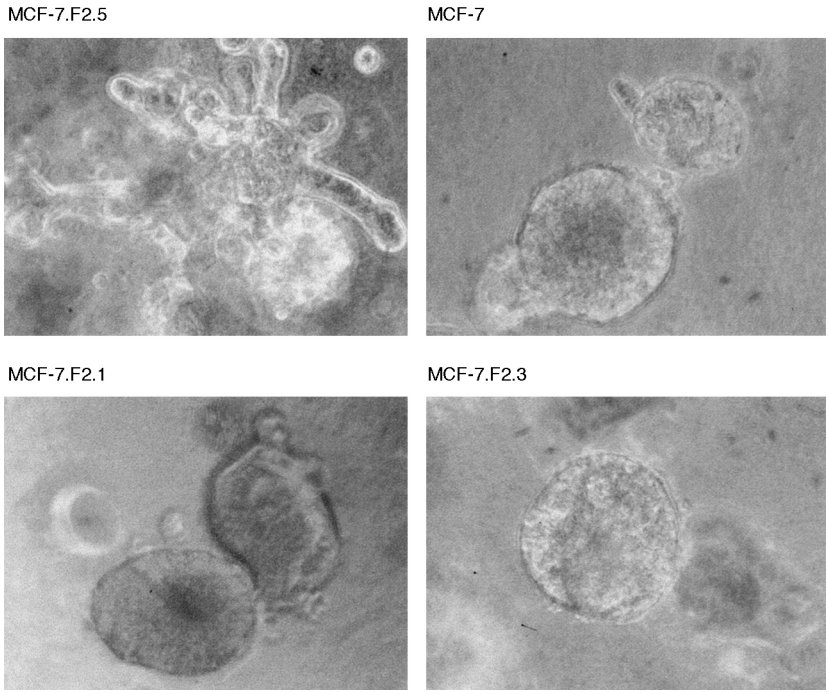

Fig. 3. Branching morphogenesis of FGF2-infected cells, MCF-7.F2.5. Cells were embedded in a reconstituted basement membrane matrix (Matrigel) for 3 weeks and culture medium was changed every 3 days. MCF-7.F2.5 cells showed evidence of branching morphogenesis, unlike parental MCF-7 cells, and negative control subclones MCF-7.F2.1 and MCF-7. F2.3 which formed spheroids. Photographs were taken at week 3 under phase contrast using an Axiovert 10 inverted microscope (Zeiss). Magnification ×200.

the anti-α2 integrin antibody, cells formed loosely associated clusters with poor intercellular adhesion, whereas in the presence of the α3 integrin antibody cells formed tight spheroids (Fig. 6). No morphological changes were found with the addition of the anti-α6 integrin antibody to MCF-7.F2.5 cells (Fig. 6). These results indicate that functional α2β1 and α3β1 integrins are essential for FGF2 induced branching morphogenesis. However, the ability of cells to bind to collagen and laminin was not affected by the overexpression of FGF2 in MCF-7 cells, since both parental and FGF2 infected cells showed similar binding to collagen I, collagen IV and laminin (data not shown).

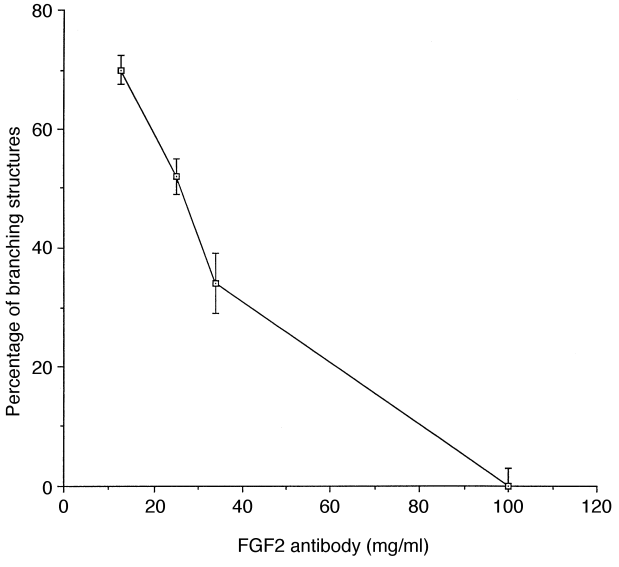

Fig. 4. Inhibition of branching morphogenesis by anti-FGF2 neutralising antibody. Serial concentrations of anti-FGF2 antibody were applied to the MCF-7.F2.5 cells cultured in Matrigel. Culture dishes containing different concentration of anti-FGF2 antibody were scored for branching structures using phase contrast microscopy at week 2. Data represent the mean percentage of branches in 200 cell clusters and standard errors are shown by bars.

3.5. Growth rate in anchorage-dependent and anchorage-independent conditions

Cell growth in monolayer culture was measured by a SRB assay (Fig. 7). The growth rate of MCF-7.F2.5 cells was slightly slower than the parental MCF-7 cells and the negative control cells, MCF-7.F2.1 and MCF-7.F2.3 in the absence of G418. Addition of exogenous FGF2 (10 ng/nl) to MCF-7 parental cells demonstrated the same inhibitory results as seen in MCF7.F2.5 cells. At day 6, the difference in the optical density value between MCF-7.F2.5, MCF-7 in the presence of FGF2 (10 ng/ml) and MCF-7 in the absence of FGF2, MCF-7.F2.1 and MCF-7.F2.3 cells was statistically significant ($P < 0.01$ by Student's t-test) (Fig. 7). Parental cells and cells infected with the *FGF2* gene were examined for their ability to form colonies in soft agar, a criterion for transformation. The *FGF2* infected cell line MCF-7.F2.5 showed a 50-fold decrease in the number of

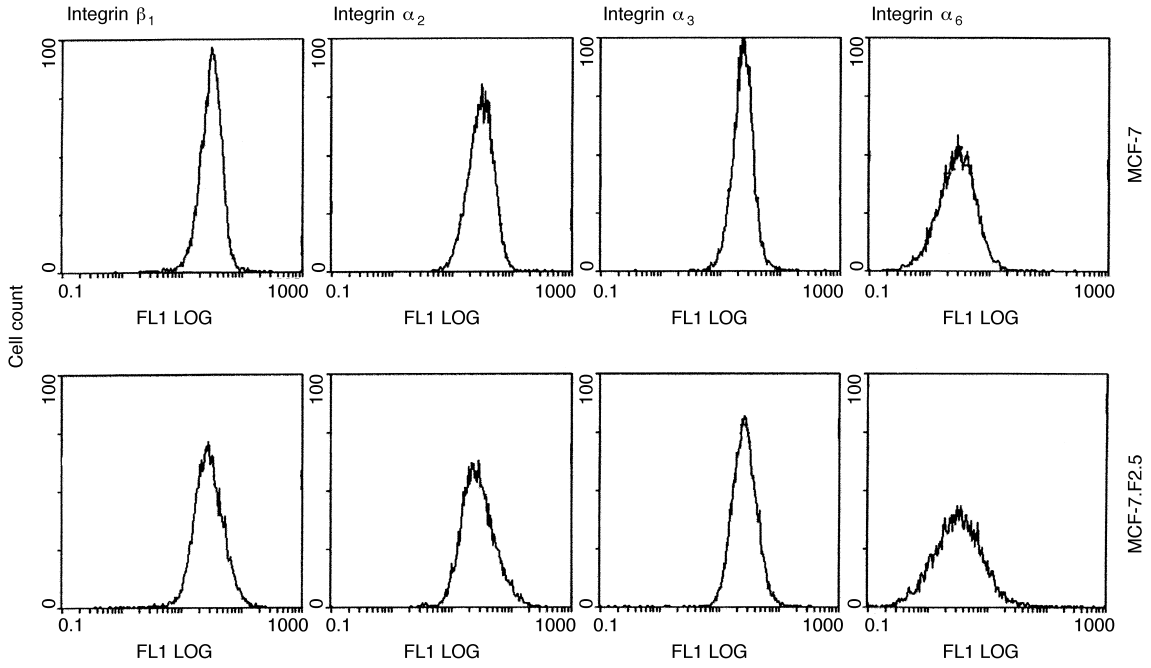

Fig. 5. Expression of β1, α2, α3, and α6 integrin subunits by FACS analysis. MCF-7 and MCF-7.F2.5 cells were incubated with anti-β1 (B3B11), α2 (P1E6), α3 (P1B5), and α6 (4F10) integrin antibodies for 1 h on ice. After PBS washes, cells were incubated with a FITC conjugated secondary antibody and then analysed using FACScan. Immunofluoresence intensity (FL1) is expressed as a log scale and there was no change in the distribution of β1, α2, α3 and α6 integrin subunits between FGF2 infected cells and parental MCF-7 cells.

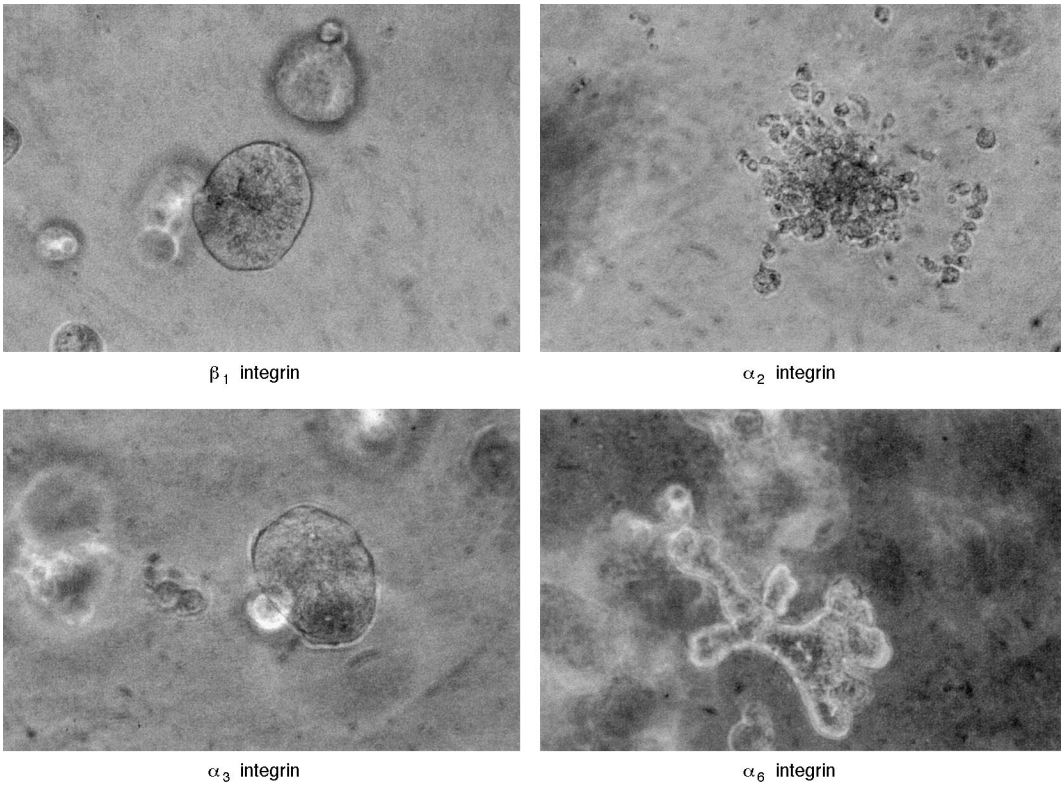

Fig. 6. Inhibition of branching morphogenesis by anti-integrin antibodies. MCF-7.F2.5 cells were embedded in Matrigel in the presence of anti-β1 (JB1a, 50 μg/ml), α2 (P1E6, 10 μg/ml), α3 (P1B5, 10 μg/ml) and α6 (NKI-GoH3, 10 μg/ml) integrin antibodies. Media containing different antibodies were changed every 3 days for 3 weeks. Untreated cells (not shown) gave rise to similar structures to those seen in Fig. 3 (see MCF-7.F2.5). Anti-β1 and α3 integrin antibodies suppressed branching morphogenesis completely whereas an antibody to α6 integrin had no effect, and the anti-α2 integrin antibody not only prevented branch formation but also inhibited intercellular adhesion. Photographs were taken using phase contrast microscopy at week 3.

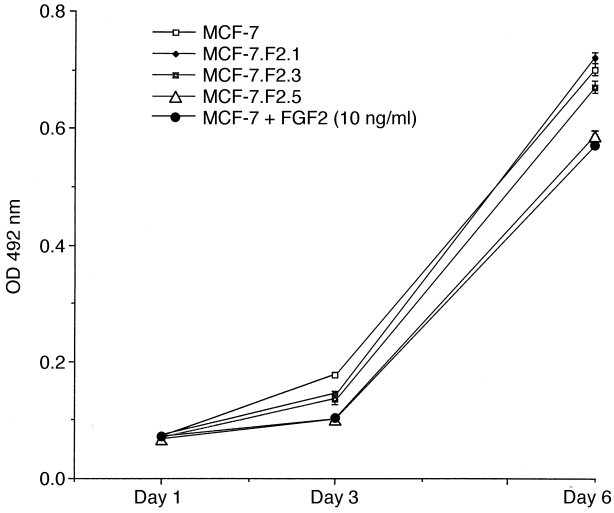

Fig. 7. Growth rate of MCF-7 and FGF2-infected subclones measured by SRB assay. Values obtained represent the mean of six replicate wells at days 1, 3 and 6 of culture and standard errors are shown by the bars. MCF-7.F2.5 and MCF-7 cells treated with FGF2 (10 ng/ml) grew more slowly than MCF-7 untreated and MCF-7.F2.1 and MCF-7.F2.3 cells. (*$P <$ 0.001 for MCF-7.F2.5 versus MCF7 by Student's *t*-test).

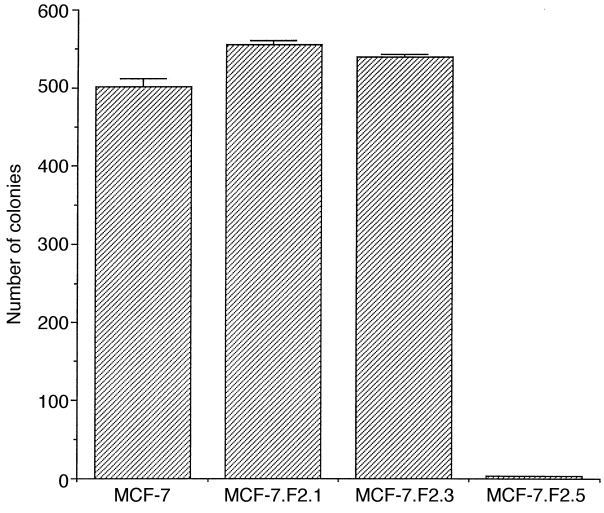

Fig. 8. Anchorage-independent growth determined by soft agar assay. 1000 MCF-7 MCF-7.F2.1, MCF-7.F2.3 and MCF-7.F2.5 cells were mixed with equal volumes of 0.6% agar in the presence of 2× growth medium and 20% FCS. The number of colonies in soft agar was counted on day 14. A cell cluster containing over 30 cells was counted as a colony. Values obtained represent the mean number of colonies from three replicate wells and standard errors are shown by the bars. A substantial reduction in the size and number of cell clusters was found with MCF7.F2.5 cells.

colonies formed in comparison with the MCF-7 parental cells and the negative control cells, MCF-7.F2.1 and MCF-7.F2.3 (Fig. 8).

3.6. Tumorigenicity

The size of tumours was monitored every week, and the nude mice were sacrificed when the tumour diameter reached 15 mm or at the end of 4 weeks. The mean volume of tumours from MCF-7.F2.5 cells was 1.6- to 3.2-fold smaller than those from MCF-7 cells (Fig. 9) during the four-week period. The difference was statistically significant ($P < 0.001$, Student's *t*-test). Histological examination revealed that tumours from *FGF2* infected cells were less mitotic than the parental cell line MCF-7. There was no morphological difference between the tumours derived from MCF-7 and MCF-7.F2.5 cell lines. Both cell lines were poorly differentiated and scored as grade III.

Blood vessel density was evaluated by counting the number of CD31 positive vessel structures. The average vessel number per high power field (200× magnification) for MCF-7.F2.5 cells was 30.5±3.5, whereas for parental MCF-7 cells was 28.5±2.75. ($P \geqslant 0.05$, Student's *t*-test). Therefore there was no increase in the vascularity of tumours formed with *FGF2* infected cells.

3.7. ERα expression in MCF-7/FGF2 transfectants and parental cells

Since both cell lines were grown inside ovariectomised nude mice in the presence of oestradiol pellets, the expression of the oestrogen receptor was examined in the resulting tumours. All tumours (100%) derived from parental MCF-7 cells expressed ERα, with the number of positive cells varying between 50 and 95%. In contrast, only 30% tumours derived from the MCF-7.F2.5 cell line expressed more than 50% ERα positive cells

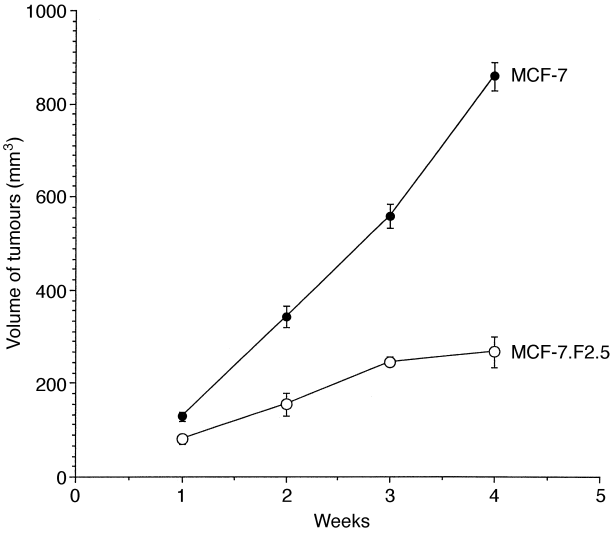

Fig. 9. Growth of MCF-7 and MCF-7.F2.5 cells in nude mice. 20 ovariectomised nude mice in each group were subcutanously injected with 10^7 cells on one flank, 6 days after implantion of oestradiol pellets (1.5 mg/60 day release). Tumours were measured every week. Values represent mean tumour volume ($n = 20$) per weekly time point and standard errors are shown by bars. FGF2-transduced cells grew significantly more slowly than parental cells ($P <$ 0.001 by Student's *t*-test).

(Fig. 10a), indicating that FGF2 down-regulated ERα expression *in vivo*. χ^2 tests showed that the difference in the number of cells expressing ERα in tumours from the two cell lines was statistically significant ($P < 0.001$). Immunocytochemical staining of monolayer cultures of MCF-7 and MCF-7.F2.5 cells also showed a reduction in the number of cells staining for ERα from 90 to 100% for parental cells down to between 15 and 20% for *FGF2* transfected cells (Fig. 10b). Furthermore, the expression of ERα transcripts was analysed by northern blotting. The expression of the 6.4 kb ERα transcript in MCF-7 *FGF2* retroviral infected cells was remarkably decreased in comparison with the parental cells MCF-7 and the negative control cells MCF-7.F2.1 (Fig. 10c). This result was corespondent with the immunochemical staining *in vitro* and *in vivo*.

4. Discussion

The growth and development of cancer is not only determined by tumour cells themselves, but also by the

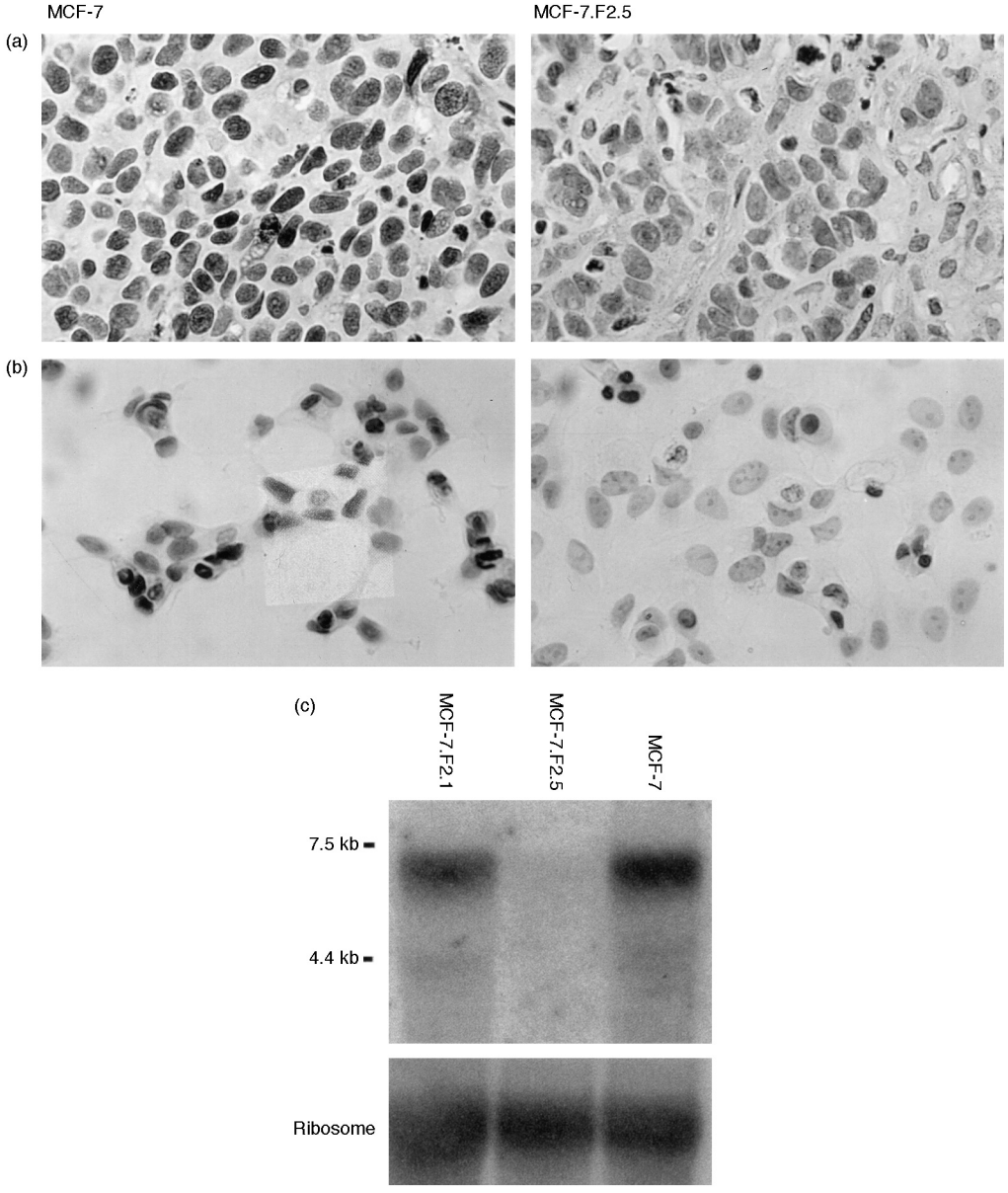

Fig. 10. Expression of oestrogen receptor alpha: (a) paraffin section of tumours from nude mice injected with either MCF-7 or MCF-7.F2.5 cells was stained with anti ERα antibody by avidin–biotin-complex indirect immunoperoxidase method; (b) MCF-7 and MCF-7.F2.5 cells cultured on four-well slides and stained with the ERα antibody using same method; (c) northern blot analysis of ERα expression. 20 μg of RNA from MCF-7, MCF-7.F2.1 and MCF-7.F2.5 cells was electrophoresed and transferred on to Hybond-N filter. After hybridisation with ^{32}P labelled dCTP ERα probe, autoradiography was performed by using film at −70°C. The equal loading of RNA was confirmed by rehybridisation of the filter with the human ribosomal probe.

microenvironment including various host cells and their products such as growth factors and cytokines. In the normal breast, FGF2 is predominantly expressed and released by myoepithelial cells [17], which are considered to be part of the host defence against cancer [32], whereas malignant tumours lose their myoepithelial cells and their derived FGF2. In this study, we demonstrated that in addition to forming organised branching structures, introduction of FGF2 into MCF-7 cells reduced their rate of proliferation on plastic, suppressed colony formation in soft agar and dramatically decreased tumorigenicity in nude mice. The release of FGF2 and response to FGF2 by MCF-7.F2.5 cells via an autocrine loop may resemble the paracrine effect of myoepithelial cell-derived FGF2 acting on epithelial cells in the normal breast. Taken together, our results may, at least partially, explain the mechanism of myoepithelial defence against breast cancer.

It has been known that the G1 phase of cell cycle is regulated by external factors such as growth factors, whereas later events in the cycle are more autonomous. In MCF-7 cells, addition of FGF2 upregulated the proteins responsible for the transition from G1 to S phase of the cell cycle such as cyclins D1 and E and cyclin-dependent kinase 4 (cdk4), also decreased cyclin A and increased p21WAF1/CIPI which resulted in an inactivation of cdk2 and dephosphorylation of the retinoblastoma protein (Rb). The net effect was an accumulation of MCF-7 cells in G0-G1 [23,33]. Presumably the effect of FGF2 on cell arrest at G1 phase may also occur in *FGF2* retroviral infected MCF-7 cells in the present study.

One of the key differences between transformed cells and normal counterparts is that transformed cells can grow while in suspension whereas non-transformed cells require adherence to a substratum. Recently it has become clear that anchorage dependence of cell growth is confined to G1, and that once cells pass a point late in G1 (termed the restriction point), they no longer require adhesion to a substratum to complete the cycle [34]. The restoration of anchorage dependence to the MCF7.F2.5 cell line may be due to the effect of FGF2 on limitation of cells passing beyond the G1 phase.

Moreover, since all nude mice were ovariectomised and implanted with the same amount of oestradiol pellets, suppression of tumorigenicity may also result from reduction of responsiveness to oestradiol due to downregulation of oestrogen receptor. In this study, we have described the downregulation of ERα expression by the *FGF2* infected cell line MCF-7.F2.5 *in vitro* and *in vivo*. A recent study demonstrated that although the mouse mammary tumour virus *Wnt*-1 transgene induced mammary gland hyperplasia and tumorigenesis in ERα null mice, the onset of tumour formation was significantly delayed and the extent of hyperplasia was restricted, indicating the importance of ERα in murine breast tumour progression [35]. Other factors that have been found to modulate ER levels include oestrogen [36] which acts directly via negative regulating promoter elements as well as at a posttranslational level. Insulin, erb-B2 ligands and progesterone also downregulate ER expression [37]. This suggests that a common signalling pathway may regulate ERα expression in breast cancer cells. Further experiments will elucidate the mechanism involved, and whether the effect of FGF2 on ER content explains any of our observations with the *FGF2*-transduced MCF-7 cells.

In general, there is a tendency that overexpression of FGF2 in a certain type of cancer tissue is correlated with a growth stimulatory effect in the same type of cancer cell line *in vitro* and *in vivo*. However, there are several exemptions. In the *FGF2* negative rat pancreatic cancer cell line, AR4-2J, although exogenously added *FGF2* stimulated their growth, the introduction of the FGF2 gene by retroviral infection led to a dramatic reduction in tumour growth in nude mice, with cells synthesising the higher molecular weight forms giving rise to only small palpable nodules [38], which was in contrast to the reports of overexpression of FGF2 in human pancreatic cancer [39]. Similarly, despite the reduction of cell growth *in vitro* and *in vivo* by *FGF2* antisense transfection in prostate cancer cell lines [40], introduction of the *FGF2* gene into a prostate cancer cell line (LNCap) did not promote tumour progression [41]. However, it seems likely that breast is the only tissue with demonstrated downregulation of *FGF2* in cancer. Our present results together with Wieder and associates [42] were able to demonstrate an inhibition of proliferation of MCF-7 cells when infected with the *FGF2* gene and following treatment of parental cells with this growth factor, which is correspondent with our previous study of the loss of *FGF2* mRNA and protein in breast cancer.

In normal breast, there is a well-controlled balance between cell division, cell differentiation and cell death. Growth arrest is coordinated with expression of a differentiated phenotype and maintenance of normal architectures. In breast cancer, cells have lost the control of growth and organisation of lobuloalveolar structures. After retroviral infection of *FGF2* gene, MCF-7 cells regained branching structures in Matrigel which resembled lobular structures in normal breast, indicating the reversal of malignant phenotype. Our preliminary study by time-lapse videomicroscopy revealed that when MCF-7.F2.5 cells were cultured on top of Matrigel, within 16 h cells moved together to form long cell cords, which connected with each other like a net, but this was not observed in MCF-7 parental cells. This structural organisation was due to cell migration which was not accompanied by cell division (data not shown). FGF2 may attract cells towards each other by providing chemotactic signals [43]. Our find-

ings are consistent with the hypothesis that, in the normal breast, myoepithelial cell-derived FGF2 provides directional signals for luminal epithelial cells.

The organisation of mammary epithelial cells into branching ducts and alveoli depends on the interplay between growth factors and cell adhesion molecules. The importance of integrins in mammary morphogenesis has been documented extensively [44]. However, the present study is the first report to describe the importance of both integrins and FGF2 in mammary morphogenesis. The inhibition of branching structures by neutralising antibodies against FGF2 and to β1, α2 and α3 integrins, suggesting that branching formation of MCF-7.F2.5 cells is dependent on both the presence of extracellular FGF2 and α2β1 and α3β1 integrins. It has been demonstrated that FGFR is capable of co-localising with integrins within focal adhesion complexes (FACs) in endothelial cells. This form of spatial integration of growth factor receptor, integrins and signal transduction molecules at the cell surface may, at least in part, explain how cells can sense multiple stimuli and generate a single, concerted response [45]. FGF2 can also collaborate with the integrin stimulated activation of the ERK class of MAP kinases in fibroblast cells once integrins are both aggregated and occupied by specific ligands [46]. These findings together with our results raise the possibility that FACs might bridge integrins and FGFR and activate downstream signalling pathways.

In conclusion, in MCF-7.F2.5 cells the acquisition of the ability to form differentiated structures in Matrigel, the loss of ability to grow in soft agar as well as reduction of tumorigenicity in nude mice suggest that FGF2 plays a tumour suppressor role in breast tumorigenesis. This may throw light on new strategies for intervention of this disease in the future.

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