Differential expression of FGF family members in a progestin-dependent BT-474 human breast cancer xenograft model

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Summary. Members of the fibroblast growth factor (FGF) family have been associated with tumor progression and angiogenesis, though the mechanism through which they affect the progression of breast cancer remains elusive. We recently showed that progestins increase the production of the potent angiogenic factor VEGF in an in vivo BT-474 human breast cancer cell-derived xenograft model. In this study we sought to determine the effect of progesterone (P) on regulation of specific FGF family members (FGF-2, FGF-4 and FGF-8) in the same model. Using immunohistochemistry we found that treatment with P significantly reduced FGF-2 and FGF-8 levels, while modestly increasing the levels of FGF-4 in tumors collected at the termination of the study or soon after P treatment began. The in vivo observations with FGF-2 were confirmed in cultured BT-474 cells, though the P-mediated reduction in FGF-2 was not blocked by the anti-progestin RU-486, suggesting that classical progesterone receptors (PR) are not involved in FGF-2 down-regulation. Also, P did not affect levels of FGF-2 mRNA in BT-474 cells, indicating that P exerts its effects on FGF-2 post-transcriptionally. Our observations suggest that the in vivo stimulation of BT-474 cell growth by P is associated with down-regulation of FGF-2 and FGF-8. Furthermore, since FGF-4 levels increased during P-treatment, FGF-4 may be required for tumor growth and maintenance and might therefore be a potential therapeutic target through which to suppress P-dependent tumor growth.

Key words: Breast cancer, Fibroblast growth factor, Progesterone, Vascular endothelial growth factor

Introduction

The vertebrate fibroblast growth factor (FGF) family is composed of twenty two structurally related polypeptides which, in the presence of heparin sulfate, bind with different affinities to four tyrosine kinase receptors (Luqmani et al., 1992; Ornitz et al., 1996; Zhang et al., 2006). Several members of the FGF family play vital roles during embryogenesis by promoting cell proliferation and migration. In adults they are equally important as regulators of tissue response to injury and in promoting angiogenesis (Ornitz and Itoh, 2001). The potent mitogenic and angiogenic potential of certain members of this family of growth factors is strongly associated with the development of solid tumors, mediated through cell proliferation and neo-vascularization under hypoxic conditions. However the role of the various members of the FGF family in human breast cancer development remains controversial.

Over-expression of FGF-2 has been described in a variety of cancers. However in studies of breast cancer there are conflicting reports regarding FGF-2 expression and its association with patient prognosis (Colomer et al., 1997; Yiangou et al., 1997; Faridi et al., 2002). The mechanisms by which FGF-2 might contribute to the pathogenesis of neoplastic processes is not well understood, though in vitro studies using T47-D cells show that in the regulation of HIF-1α activation and VEGF expression, hypoxia and FGF-2 exert an additive effect (Shi et al., 2007). Aberrant FGF-4 gene expression has been documented in different sets of breast tumors

(Theillet et al., 1989; Schmitt et al., 1996) but clear associations with the pathogenesis of human breast cancer have not been found and FGF-4 is not commonly expressed in human breast tumors. Previously, Hajitou et al. (2000) found that FGF-4 exerts effects that are angiogenic, since it promotes the secretion of VEGF by MCF-7 breast cancer cells. Over-expression of FGF-8 has been associated with the pathogenesis of other neoplastic processes, such as prostate cancer. FGF-8 is also expressed in normal breast tissue and has been shown to be over-expressed in both the lactating mammary gland and in breast tumors (Tanaka et al., 1998; Marsh et al., 1999; Zammit et al., 2002). FGF-2, FGF-4 and FGF-8 demonstrate increased tumorigenic potential and a capacity to promote metastasis when transfected into breast cancer cell lines that are subsequently used in xenograft studies (Bagheri-Yarmand et al., 1998; Hajitou et al., 2000; Ruohola et al., 2001). To date, several reports show a relationship between the expression of certain of these factors and tumorigenesis in different types of cancer (Ornitz et al., 1996; Ornitz and Itoh, 2001; Chaffer et al., 2007) but no specific association between members of the FGF family and breast pathogenesis.

Recent clinical trials and studies, such as the Women's Health Initiative study, suggest that there is an increased risk of breast cancer in post-menopausal women who receive combined estrogen and progestin hormone replacement therapy compared with women who receive only estrogen or placebo (Rossouw et al., 2002; Krämer et al., 2006). However, the molecular mechanism by which progestins increase breast cancer risk remains unknown. We recently established an in vivo human breast cancer cell-derived xenograft model and demonstrated that both naturally-occurring and synthetic progestins increase the progression and metastasis of tumors in this model, and furthermore, that tumor development is dependent on the production of VEGF by tumor cells (Liang et al., 2007, 2010). Since FGF family members have been shown to act synergistically in the presence of VEGF and also to increase VEGF secretion under conditions of hypoxia (Shi et al., 2007), we conducted studies aimed at determining the influence of progesterone (P) on the expression of FGF-2, FGF-4 and FGF-8 in BT-474 human breast cancer cells, both in vitro and in vivo in the xenograft model. Our xenograft studies included an analysis of the effect of in vivo exposure to P in both the end point tumor samples (day 45-50 after P treatment) and in tumor samples that were exposed to P for a brief period (3 days), to determine whether the effects of P are more immediate during the early growth phase of xenografts. Using immunohistochemistry to localize protein expression, it was apparent that FGF family members are differentially regulated in the model tested; FGF-2 and FGF-8 were lower, while levels of FGF-4 were elevated in end-point samples. Interestingly, FGF-2 and FGF-8 levels were also drastically reduced in the early samples, while FGF-4 showed modest up-

regulation. Implications of the results are discussed.

Materials and methods

Reagents

Progesterone and RU-486 were obtained from Sigma-Aldrich (St. Louis, MO). FGF-2 antibody (SC-79) and anti-ß-actin antibody were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, Ca). FGF-4 (ab65974) and FGF-8 (ab81384) antibodies were obtained from Abcam (Cambridge, MA).

Cell culture and treatments

BT-474 breast cancer cells were grown in phenol red-free Dulbecco's modified eagle medium (DMEM/F12; Invitrogen, Carlsbad, Ca) supplemented with 10% fetal bovine serum in 100 mm cell culture plates. Once cells had reached approximately 50-60% confluence they were washed and cultured for 24 hours in DMEM/F12 supplemented with 5% dextran coated charcoal (DCC). DCC media was then replaced and cells were treated with either progesterone at concentrations of 10, 100 or 1,000 nM, progesterone receptor antagonist RU-486 at a concentration of 1 μM, or a combination of both for 12 h, after which cells were washed and collected using 0.05% trypsin. Control groups were treated with ethanol. When using combined treatment RU-486 was added 30 minutes prior to progesterone supplementation.

FGF-2 Western blot analysis

Whole cell extract was made from BT-474 cells and protein aliquots (50 μg) from each group were separated by gel electrophoresis (NuPAGE 10% Bis-Tris Gel, Invitrogen, Carlsbad, CA). Electrophoresis was performed at 120 V for 1.5 h using NuPAGE MES-SDS running buffer. Separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA) at 35 V for 1.5 h, and blots were subsequently blocked in 5% nonfat dry milk in tris-buffered saline containing 1% Tween-20 (TBS-T) for 1 h at room temperature. Membranes were incubated with primary FGF-2 antibodies (1:200) for 2 h at room temperature and then with secondary rabbit antibody (1:2000) for 1 h also at room temperature. Immuno-reactive bands were visualized using an ECL Plus detection kit (Amersham, Pharmacia Biotech, Arlington Heights, IL). Membranes were stripped and re-blotted for ß-actin (Sigma-Aldrich, St. Louis, MO), as a control for protein loading. The experiment was conducted twice.

RT-PCR

Primers for FGF-2 (Forward 5'-GGCTTCTT CCTGCGCATCCA-3', Reverse 5'-GCTCTTAGCAG

ACATTGGAAGA-3'; Barclay et. al., 2005) were synthesized by IDT (Coralville, IA). Total RNA was extracted from cultured cells (treated with hormones and antagonists in DCC supplemented media for 6h) using RNAzol® RT (Molecular Research Center inc., Cincinnati, OH) according to the manufacturer's protocol. RT-PCR amplification was performed in a thermocycler using Invitrogen superscript III one step RT-PCR amplification kit (Invitrogen, Carlsbad, Ca). RT-PCR conditions: 60°C for 30 min; 94°C for 2 min; 40 cycles of 94°C for 15 sec, 55°C for 30 sec, and 68°C for 60 sec; final elongation at 68°C for 5 min. Primers for VEGF analysis are described in our earlier publication (Hyder et al., 2001). Products were analyzed by electrophoresis on a 1.5% agarose gel with ethidium bromide. Results are derived from 3 separate experiments.

Xenograft sample selection

BT-474 xenografts were grown as previously described using protocol that was approved by the institutional animal care and use committee (Liang et al., 2007). For immunohistochemical analysis of FGF expression in xenograft tumors, tumor samples were collected from P treated and placebo treated animals at the end of the experiment (T) as described previously (Liang et al., 2007). Samples were also collected 3-days post P treatment (E) to determine whether P-mediated effects observed at later stages actually occurred early following P administration (Fig. 1).

Immunohistochemical analysis

Previously fixed (paraformaldehyde 4%), paraffin embedded xenograft tumors were sectioned (5 um) and mounted onto ProbeOn Plus microscope slides (Fischer Scientific, Inc., Pittsburg, PA). Sections were dewaxed in xylene, rehydrated through graded concentrations of ethanol, rinsed in distilled water, and, if necessary, stored in PBS at 4°C until use. Sections were subjected to heat-induced epitope retrieval. Slides were treated with 3% hydrogen peroxide in absolute methanol (to inactivate endogenous peroxidase activity), washed in 3% PBS, incubated in blocking buffer with 5% bovine serum albumin for 20 min, and treated with the primary polyclonal antibody at room temperature for 60 min. Sections were then washed and incubated with EnVision+, a horseradish peroxidase labeled polymer conjugated with anti-rabbit antibodies (DAKO-Carpinteria, CA). Bound antibodies were visualized with 3,3'-diaminobenzidine tetrahydrochloride (0.05% with 0.015% H_2O_2 in PBS; DAKO, Carpinteria, CA). Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared, and cover-slipped for microscopic examination. Using morphometric software (FoveaPro 3.0, Reindeer GraphicsAsheville, NC) immunohisto-chemical staining was quantified in each group from four tumor pictures recorded from three different tumor

sections at 20x magnification. Results are expressed as stained pixel area.

Statistics

Statistical analysis was performed using SigmaStat® Software version 3.5 (Systat Software, San Jose, CA). Groups were analyzed using t-test. When normality failed the Man-Whitney test was used. For statistical significance p values <0.05 were considered significant.

Results

Progesterone suppresses FGF-2 and FGF-8 and increases FGF-4 protein levels in BT-474 xenografts

Fig. 1 illustrates a typical result from a progestin-induced BT-474 xenograft experiment and is taken and modified from a previously published manuscript with permission (Liang et al., 2007). In this model tumors demonstrate an initial growth phase, which is followed by a reduction in tumor growth over time. However, the addition of progesterone pellets during the regression phase causes a resumption of tumor growth. In order to determine alterations in the levels of three important members of the FGF family within xenografts, we collected tumor samples both at the termination of the experiment (T) and also 3 days after progesterone supplementation (E). The E samples were obtained in
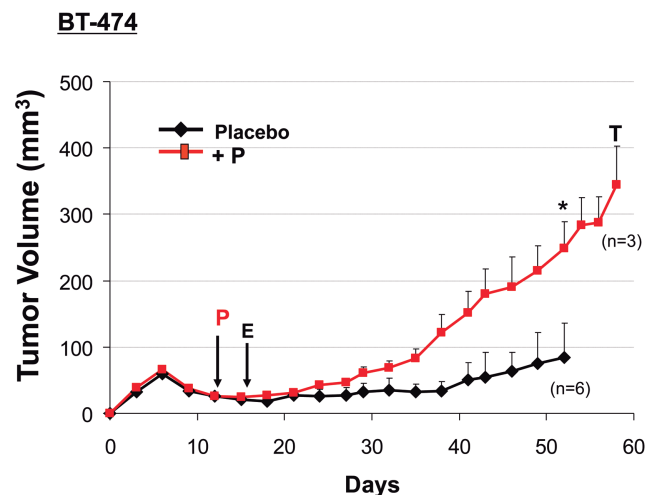
BT-474

Fig. 1. Progesterone (P) promotes the growth of BT-474 xenografts (modified from Liang et al. 2007 with permission). BT-474 xenografts demonstrate an initial tumor growth phase, followed by a decline within 5-7 days. Supplementation of P pellets causes a resumption in tumor growth. Tumors were collected at an early P-supplemented growth phase (E) and at the termination of the experiment (T). Red line represents tumor volumes following P supplementation and samples taken from this group represent the +P group in subsequent figs. Black line represents tumor volumes without P supplementation and samples taken from this group are referred to as -P samples. All tumor cells were implanted into mice following implantation of estrogen pellets.

order that we might assess early P-mediated changes in protein levels of FGF family members using immunohistochemistry. The T samples showed reduced levels of expression of both FGF-2 and FGF-8 when tumors were progressing in response to P compared with those that were exposed to estradiol alone (Fig. 2). However, levels of FGF-4 expression increased in the same samples. By analyzing 3-day post P samples (E) we sought to determine whether alterations in the levels of different FGF family members occurred early
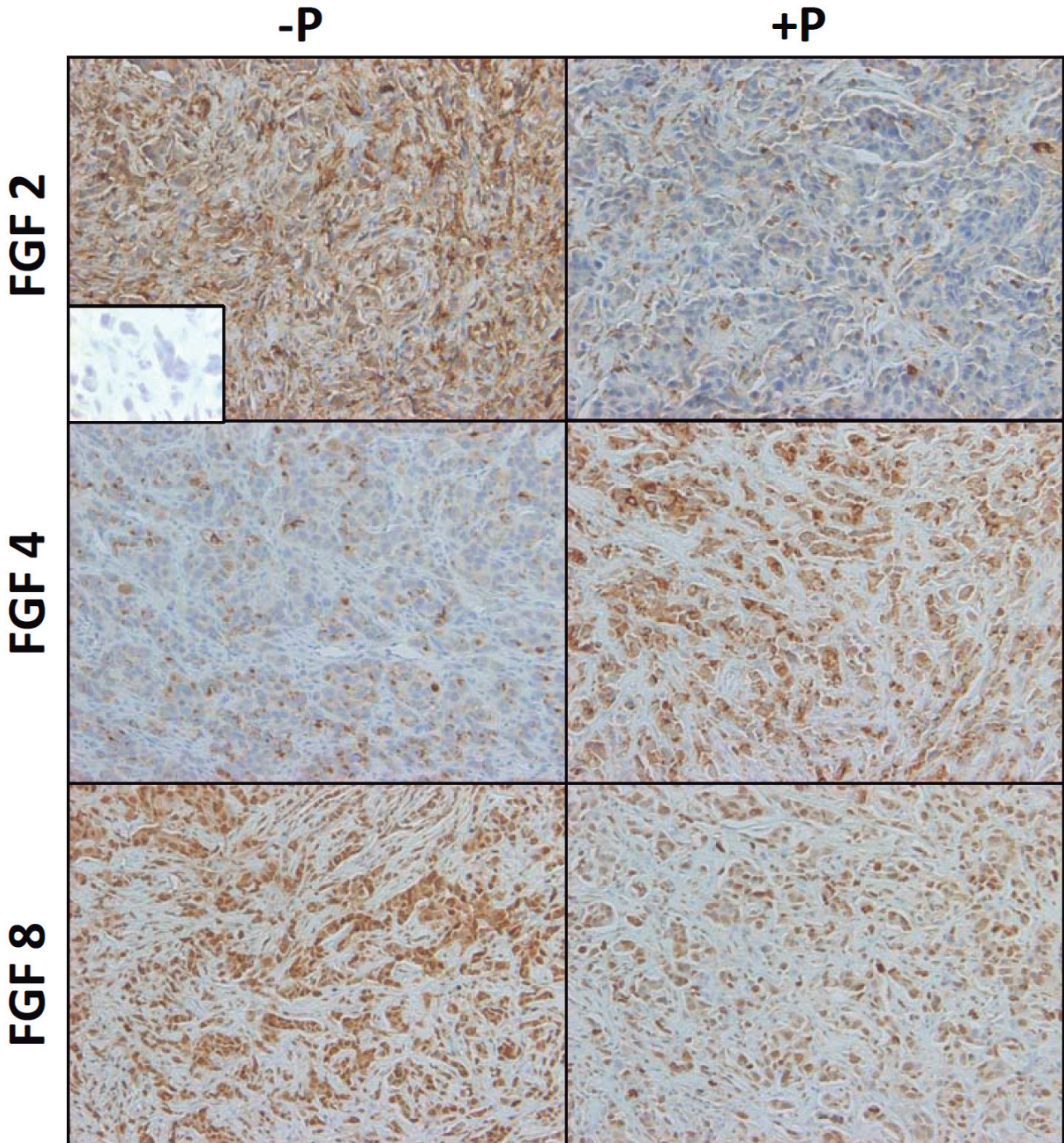

Fig 2. Immunohistochemical analysis of FGF-2, FGF-4 and FGF-8 expression in BT-474 xenografts in the absence (-P) and presence (+P) of progesterone at the termination of the experiment. Insert shows negative control for antibody staining. x 20
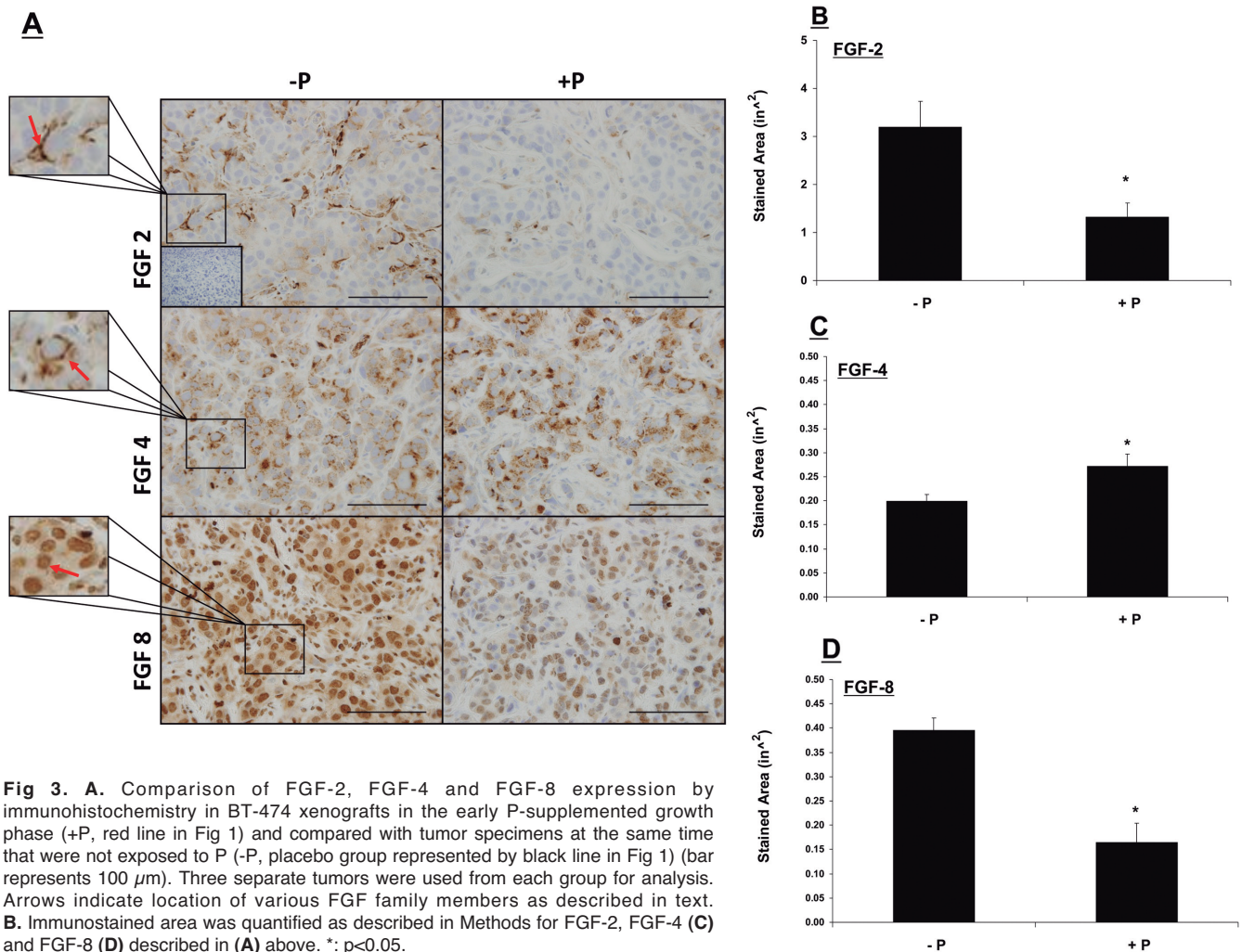
following P exposure. Fig. 3A demonstrates that early events in P-mediated xenograft progression include reduced levels of FGF-2 and FGF-8, as well as increased FGF-4. In Figs. 3B-D the quantitative data obtained from E samples is presented, showing significant differences in the levels of FGF-2, -4 and -8 in tumor sections. These observations demonstrate that changes observed in the expression levels of FGF family members occur early following P treatment and perhaps represent a direct effect of progesterone exposure.

There were no differences in cellular localization of FGF family members in E or T samples, either following exposure to P, or when P was absent from the experiment. FGF-2 was expressed in both the stromal and epithelial compartments. Stromal cells demonstrated very strong cytoplasmic staining for FGF-2, while in the epithelial compartment FGF-2 staining in the cytoplasm varied from weak to strong. Overall, solid epithelial tumors possessing a reduced stromal component showed a tendency towards lower FGF-2 expression compared

with those tumors that had a higher stromal component. We found that FGF-4 was expressed in the cytoplasm of BT-474 cells, while FGF-8 expression was primarily nuclear, with faint staining of the cytoplasm.

Progesterone suppresses FGF-2 levels in BT-474 cells in vitro

Since FGF-2 levels were dramatically suppressed in E samples following P treatment, we performed cell culture studies using BT-474 cells, aimed at determining whether P directly inhibited its expression via PR. Incubation of BT-474 cells with P (10 nM) for 12 h reduced FGF-2 levels (Fig. 4A), however a 100-fold excess (1 μM) of the anti-progestin RU-486 failed to reverse this suppression, indicating that nuclear PR were not involved in mediating the effects of P. RU-486 alone (1 μM) showed marked suppression of FGF-2 in BT-474 cells, a finding that is similar to in vivo observations in other progestin-models, in which RU-486 functions as

Fig 3. A. Comparison of FGF-2, FGF-4 and FGF-8 expression by immunohistochemistry in BT-474 xenografts in the early P-supplemented growth phase (+P, red line in Fig 1) and compared with tumor specimens at the same time that were not exposed to P (-P, placebo group represented by black line in Fig 1) (bar represents 100 μm). Three separate tumors were used from each group for analysis. Arrows indicate location of various FGF family members as described in text. **B.** Immunostained area was quantified as described in Methods for FGF-2, FGF-4 **(C)** and FGF-8 **(D)** described in **(A)** above. *: $p<0.05$.

an agonist of PR and mediates its effects via membrane PR (Bottino et al., 2011; Fjelldal et al., 2010). Interestingly, P reduced FGF-2 levels in a concentration-dependent manner and, at a level of 1 uM, was similar in its capacity to suppress levels of FGF-2 as RU-486 (Fig. 4A). These observations suggest that P-mediated suppression of FGF-2 likely occurs via extra-nuclear PR and, quite likely, membrane PR in BT-474 cells.

Progesterone does not suppress FGF-2 at the transcriptional level

In order to determine whether P suppressed FGF-2 at the transcriptional level, we treated BT-474 cells for 6 h with 100 nM P, collected RNA from treated cells, and amplified RNA in order to detect FGF-2 message. No changes in FGF-2 expression were present at the RNA level, either with or without P treatment, suggesting that P-mediated changes in FGF 2 expression occur at the protein level (Fig. 4B). To ensure that the ligands were functional. The same set of RNA was also assessed for expression of VEGF, a gene whose transcriptional response to P is well characterized (Hyder et al., 2001). As shown in a representative example in Fig. 4C, P

induced VEGF in BT-474 cells and this induction was blocked by the inclusion of anti-progestin RU-486.

Discussion

The use of combined estrogen/progestin hormone replacement therapy in post-menopausal women has been associated with an increased risk of breast cancer, compared with those receiving estrogens alone or placebos (Rossouw et al., 2002; Krämer et al., 2006). Our previous studies showed that P increases VEGF in breast cancer cells; this may be one mechanism by which the disease progresses in women who have been administered P (Liang et al., 2007). However, the role of other growth factors that may also be under P control remains elusive. The FGF family is a group of proteins that are not only essential for development, proliferation and survival of tumor cells (Ornitz et al., 1996; Ornitz and Itoh, 2001; Chaffer et al., 2007), but have also been shown to interact with VEGF (Shi et al., 2007). Consequently, this study was designed in order to determine the influence of P on three FGF family members, FGF-2, FGF-4 and FGF-8, since these proteins are associated with angiogenesis and breast
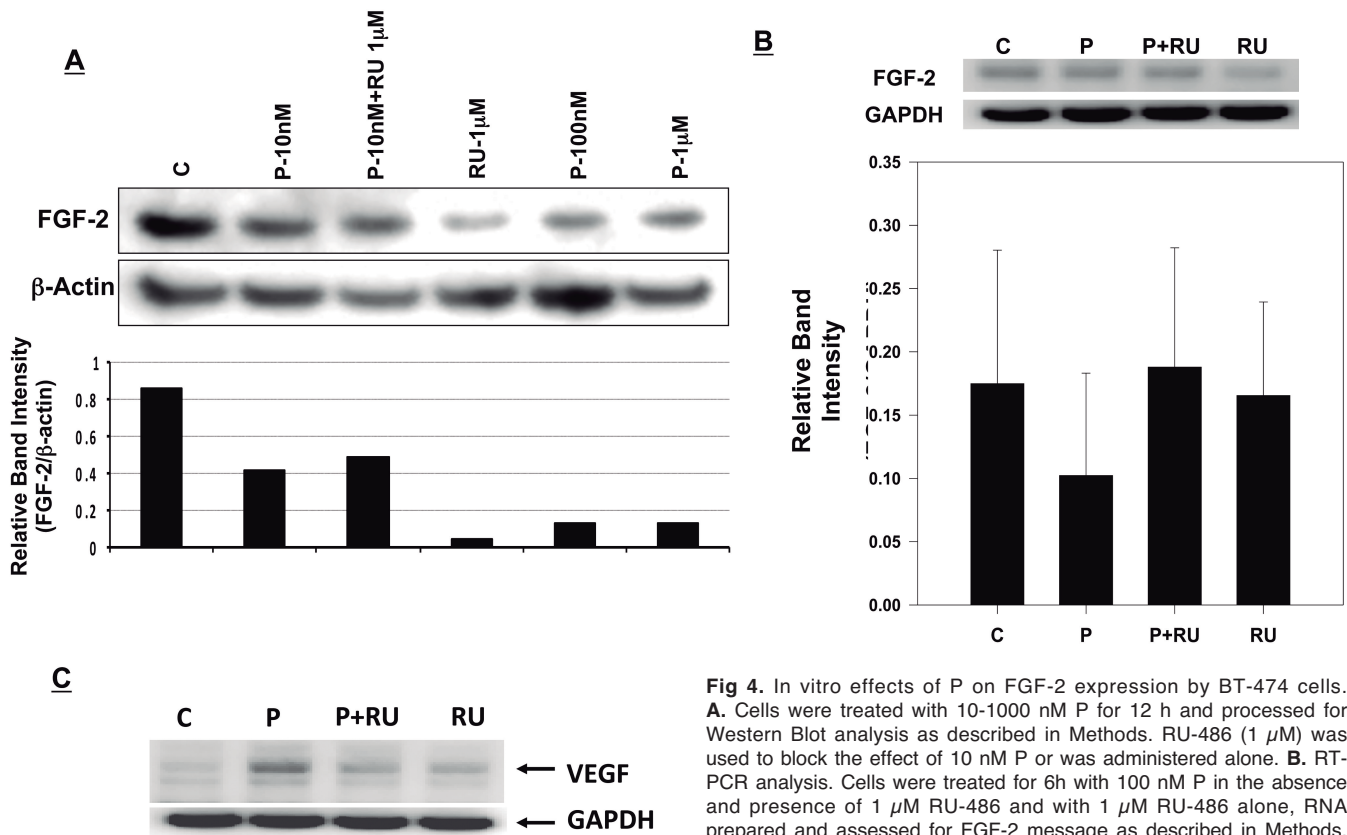

Fig 4. In vitro effects of P on FGF-2 expression by BT-474 cells. **A.** Cells were treated with 10-1000 nM P for 12 h and processed for Western Blot analysis as described in Methods. RU-486 (1 μM) was used to block the effect of 10 nM P or was administered alone. **B.** RT-PCR analysis. Cells were treated for 6h with 100 nM P in the absence and presence of 1 μM RU-486 and with 1 μM RU-486 alone, RNA prepared and assessed for FGF-2 message as described in Methods. Blots were scanned and normalized to GAPDH message. Mean of three determinations is plotted. **C.** A representative example of RT-PCR analysis for VEGF expression in samples taken from **B**.

tumor progression (Bagheri-Yarmand et al., 1998; Hajitou et al., 2000; Ruohola et al., 2001; Shi et al., 2007; Schwertfeger, 2009; Hynes and Watson, 2010). We found that in vivo P differentially influences the expression of FGF family members in our P-dependent tumor progression model. P reduces the expression of FGF-2 and FGF-8 but induces FGF-4 expression, suggesting that P-dependent progression of BT-474 cells in vivo may involve selective expression of particular FGF species in breast tumors. Since FGF-4 was elevated in tissues obtained at an early stage of the study, as well as in tissues collected at the end of the experiment, we propose that it plays a proliferative role in the P-dependent xenograft model and that it likely is essential for tumor progression. We might speculate that increased levels of FGF-4 may interact with P-induced VEGF to promote breast cancer cell progression or that it may be required for sustained VEGF production from tumor cells (Hajitou et al., 2000), reducing the necessity of continued FGF-2 and FGF-8 production. Future studies using FGF-4 specific antibodies will help determine the specific role of FGF-4 in the progression of tumor development in BT-474 xenografts. In addition, studies in which FGF-4 can be regulated in vivo in tumor cells using regulatable siRNA vectors will also shed light on whether FGF-4 is essential for tumor progression following P treatment.

FGF-2 is a ubiquitously expressed growth factor in mammalian tissues and is considered a potent mitogenic and angiogenic factor. It is produced by many different types of cell, including epithelial cells, fibroblasts, macrophages and endothelial cells (Luqmani et al., 1992; Levin et al., 2004; Chaffer et al., 2007). In the normal human mammary gland it is expressed in myoepithelial and epithelial cells (Luqmani et al., 1992; Bagheri-Yarmand et al., 1998; Granato et al., 2004). However, in mammary tumor cells elevated levels of FGF-2 is correlated with apoptosis (Maloof et al. 1999), suggesting that down-regulation of this growth factor may be important to promote tumor progression. Our studies showed that FGF-2 was dramatically down-regulated in both early and terminally-collected samples, indicating that this effect likely occurred as a direct result of exposure to P rather than arising due to secondary events in other signal transduction processes that influence FGF-2 levels. To further investigate the involvement of PR in mediating such early changes in FGF-2 levels, we conducted in vitro studies using cultured BT-474 cells. Incubation of BT-474 cells with 10 nM P elicited a reduction in FGF-2 expression at the protein level, an effect that was not reversed when a 100-fold higher concentration of the anti-progestin RU-486 was added to block the effects of P. Exposure of BT-474 cells to a higher dose of P further reduced the levels of FGF-2. The inability of RU-486 to suppress the effects of P suggests that P receptors in the cell membrane may be involved in mediating its effects. Indeed, such membrane receptors have been described in other progestin models in which both P and RU-486 function

as PR agonists (Bottino et al., 2011; Fjelldal et al., 2010). Interestingly, RU-486 has also been shown to function as an agonist in cells that express high levels of the PR-B isoform (Meyer et al., 1990; Wagner et al., 1996; Giulianelli et al., 2008). Although unlikely, it is also possible that the effects of high concentrations of P are mediated through other steroid receptors, such as androgen and glucocorticoid receptors, which possess low binding affinity for the ligand. This however is unlikely, since low levels of P (10 nM) were also able to reduce FGF-2 levels in BT-474 cells. Furthermore, our results detected no changes in the levels of FGF-2 transcript, suggesting that the effects of P on FGF-2 are post-transcriptional. It is also possible that FGF-2 levels are reduced not via a PR-mediated effect, but as a consequence of a secondary effect, such as inhibition via increased levels of VEGF which is also produced in response to P. Tumor cells may not need to express several growth factors at the same time and VEGF and FGF-4 may suffice for their survival and proliferation, an idea that is supported by the observation that FGF-2 is present during the initial phases of tumor growth, prior to P exposure when VEGF is also expressed in tissues (Liang et al., 2007). Following administration of P, VEGF levels increase while those of FGF-2 diminish. In advanced breast tumors FGF-2 levels have been shown to drop, possibly due to the presence of other growth factors such as VEGF compensating and contributing to angiogenesis and ultimate tumor survival (Colomer et al., 1997). In our xenograft model cells are injected without matrigel and it is unclear whether the initial interaction of BT-474 breast cancer cells with the stroma of a foreign host might contribute to the changes in FGF-2 expression. For this reason further studies are required if we are to completely elucidate the roles of FGF-2 in the P-dependent breast tumor model used in these studies.

As was the case with FGF-2, we also saw a reduction in the expression levels of FGF-8 in response to P, indicating that FGF-8 is also not essential for P-mediated progression of BT-474 cells in the in vivo tumor xenograft model. However it is also possible that down-regulation of both FGF-2 and FGF-8 may even be essential for progression of P-dependent xenografts, since over expression of FGF-2 has been linked with tumor cell apoptosis (Maloof et al., 1999). Further in vivo studies are necessary to determine whether clones which constitutively express these factors are able to progress in response to P.

In conclusion, our observations provide evidence that P differentially regulates certain important members of the FGF family in the P-dependent xenograft model. Furthermore, regulation of FGF species by P is not mediated via classical nuclear PR, a finding in direct contrast to P-mediated VEGF regulation, which does occur through the interaction of P with its nuclear receptors (Hyder et al., 1998, 2001). Our findings suggest that FGF-4 may play an important role in P-dependent tumor progression, making it a potential

target by which to control such cancers *in vivo*. We would like to speculate that suppression of both P-induced FGF-4 and VEGF-mediated biological processes by antibody-neutralization of their biological properties might provide us with a means by which to combat P-dependent breast cancer, as well as possibly preventing the emergence of drug resistant tumors. These ideas remain to be tested.

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