

RESEARCH ARTICLE

Cellular and Molecular Changes in MNU-Induced Breast Tumours Injected with PF4 or bFGF

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Abstract

Background: Angiogenic activity has been considered to reflect important molecular events during breast tumour development. The present study concerned cellular and molecular changes of MNU-induced breast tumours subjected to promotion and suppression of angiogenesis. **Methods:** Female Sprague Dawley rats at the age of 21 days received MNU at the dose 70 mg/kg of body weight by intraperitoneal injection. Three months post-carcinogen initiation, mammary tumours were palpated and their growth was monitored. When the tumour diameter reached 1.0 ± 0.05 cm, rats were given bFGF or PF4 intratumourally at a dose of 10 μ g/tumour. Entire palpable tumour were subsequently excised and subjected to histology examination, IHC staining, and RT-PCR. **Results:** No critical morphological changes were observed between pro-angiogenic factor, bFGF, and control groups. However, increase of tumour size with more necrotic and diffuse areas was notable in tumours after anti-angiogenic PF4 intervention. ER and PR mRNA expression was significantly up- and down-regulated in bFGF and PF4 groups, respectively. The trends were significantly associated with peri- and intratumoural MVD counts. However, irrespective of whether we promoted or inhibited angiogenesis, the expression of EGFR and ERBB2 continued to be significantly increased but this was not significantly associated with the MVD score. No significant differences in E-cadherin and LR gene expression were noted between intervention and control groups. **Conclusion:** ER and PR receptor expression shows consistent responses when tumour angiogenesis is manipulated either positively or negatively. Our study adds to current understanding that not only do we need to target hormonal receptors, as presently practiced, but we also need to target endothelial receptors to successfully treat breast cancer.

Keywords: MNU-induced breast tumour- PF4- bFGF

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Introduction

Angiogenesis is a growth process of new blood vessels from existing vasculature, enabling tumour cells to increase their blood supply (Folkman, 1994). Realizing this has encouraged researchers to design a strategy to prevent tumors from sending out signals to recruit new blood vessels that later inhibits tumor growth. A well-known angiogenic factor, VEGF (vascular endothelial growth factor), shows a significant role in the progression and prognosis of breast cancer (Carmeliet and Jain, 2011). Moreover, bevacizumab (Avastin), a recombinant humanized monoclonal antibody against VEGF is a current antiangiogenic treatment in breast cancer clinical trials, which has shown significant clinical outcomes in metastatic breast cancer treatment. However, the heterogeneity of breast tumor blood vessels influences the susceptibility of this antiangiogenic therapy. Therefore, understanding on the biological roles of other pro- and anti-angiogenic factors in breast cancer is crucial to further understand how the vasculature can be effectively targeted

in breast tumors.

Extensive laboratory data demonstrate the role of basic fibroblast growth factor (bFGF), a pro-angiogenic factor and Platelet Factor 4 (PF4) and anti-angiogenic factor (PF4) in tumour angiogenesis. bFGF has been found to stimulate vascularization in a synergistic action with VEGF, which increases tumour growth in vitro (Giavazzi et al., 2003b). A number of studies have explored the prognostic value of bFGF in different tumour subtypes (Granato et al., 2004; Felix et al., 2012; Jibiki et al., 2014; Hu et al., 2016). This pro-angiogenic factor has been demonstrated upregulates hepatocytes growth factor (HGF), matrix metalloproteinases (MMPs) and apoptosis-related markers (Bcl-2 and survivin) and induces tumour invasive and anti-apoptotic phenotypes (Xiao et al., 2008; Nishida et al., 2011; Eto et al., 2012; Kim et al., 2012). By contrast, PF4 regulates angiogenesis in an adverse manner by interfering bFGF and VEGF activities (Bikfalvi, 2004a; Bikfalvi, 2004b; Bikfalvi and Gimenez-Gallego, 2004; Wang and Huang, 2013). PF4 has been studied inhibits tumor progression by suppressing

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tumor-induced angiogenic activity (Maione et al., 1990; Gupta and Singh, 1994; Tanaka et al., 1997; Perollet et al., 1998). However, very little information is available for the molecular mechanism of the breast tumour growth manipulated by bFGF or PF4 intervention.

Evaluation of tumour angiogenesis via microvessel density (MVD) counting study is normally performed via immunohistochemistry staining using anti-endothelial markers like factor VIII-related antigen, CD31, CD34 and the activated endothelial cell marker CD105 (Fox and Harris, 2004; Uzzan et al., 2004). Several studies have demonstrated the role of tumour MVD as a prognostic and predictive biomarker for breast cancer (Weidner et al., 1991; Gasparini et al., 1995b; Engels et al., 1997; Guidi et al., 2000; Shimizu et al., 2000; Uzzan et al., 2004; Popiela et al., 2008). In addition, the MVD counts has been significantly correlated with ER (estrogen receptor) and PR (progesterone receptor) status of breast tumours (Vamesu, 2007; Biesaga et al., 2012; Bharti et al., 2015). High MVD counts also appears to be associated with epidermal growth factor receptors, EGFR and ERBB2, the important growth factors in breast tumour development (Weidner and Gasparini, 1994; Koukourakis et al., 2003; Blackwell et al., 2004; Carvalho et al., 2013). Elevated MVDs were also correlated with loss of E-cadherin (Gong et al., 2005) and laminin receptor (LR) (Gasparini et al., 1995a), which are responsible for breast tumour invasion, migration and metastases.

In this study, we aim to analyze the histology of MNU- induced breast tumour subjected to promotion and suppression of angiogenesis. It is important to see the changes on the histological phenotypes in breast tumor is dependent or not on the angiogenic activity. In addition, we hypothesised that different angiogenic manipulation may influence the molecular pathways of ER, PR, EGFR, HER2, E-Cadherin and LR of breast tumourigenesis. Therefore, our second objective is to investigate the expression the above receptors in MNU- induced breast tumour subjected to promotion and suppression of angiogenesis and to correlate the expression of these receptors with the degree of angiogenesis seen. Being able to understand the interactions between the above receptors' expressions associated with angiogenic activity could identify signals that can limit vascular networks and ultimately tumour growth and metastasis.

Materials and Methods

Study design

The present study involved a group of female Sprague Dawley rats. The rats were exposed to 1-methyl-1-nitrosurea (MNU) carcinogen to induce breast tumours. Subsequently, tumour growth were manipulated with pro-angiogenic factor, bFGF and anti-angiogenic factor, PF4. Rats were sacrificed a month later and the entire palpable tumours were excised. Tumour samples collected were subjected for histological assessment, IHC staining and RT-PCR. Flow-chart of study design is shown in Figure 1.

Animal study

24 female Sprague Dawley rats 20 days old were obtained from Animal House Unit at Universiti Sains Malaysia. The Animal Ethics Committee USM has approved all the procedures for the use of the experimental animals. The rats were housed and kept at the Animal House Unit. The rats were caged in groups of two in an environmentally controlled room maintained at 22 °C and 50% relative humidity with a 12-hour light/dark cycle. Each of the cages carried an identification tag, stating all required details. The rats were fed a standard laboratory diet and distilled water ad libitum. For hygienic purposes, cages were cleaned once a week.

bFGF and PF4 intervention

At the age of 21 days, all rats had induction of mammary carcinogenesis by single intraperitoneal injections with MNU at the dose of 70 mg /kg body weight (Jaafar et al., 2009). Once a tumour was detected, growth was monitored in terms of size with each breast lesion measured and recorded on alternate day. When the lesions reached a mean tumour diameter of 1.0 ± 0.5 cm, the rats were randomised into three groups (eight rats per group). The intervention of angiogenic factors was initiated by intralesional injection of bFGF and PF4 at dose of 10µg/tumour. Rats in the control group received normal saline (0.9% salt solution) only. After about a month later, all rats were sacrificed using gaseous CO₂ inhalation. All tumours were carefully excised. The fresh excised tumours from each group were partly separated into two groups; half of the tissues will be fixed in 10 % formalin and subjected for H and E staining and histopathological assessment, while other fresh tumour tissues were kept in - 80 °C for the use on molecular technique purposes.

H and E diagnosis

From paraffin-embedding block, 4 µm of sample tissue was sectioned and was stained with hematoxylin-eosin in order to examine the histopathological criteria of tumour lesions. This technique followed a standard procedure described previously (Jaafar et al., 2009).

Immunohistochemistry (IHC) staining

The IHC technique using Blood Vessel Staining kit (DAKO) was performed to demonstrate the expression of vascular endothelial marker, factor VIII-related antigen (FVIII-RA). Serial sections of 4 µm were cut from formalin fixed, paraffin embedded tissue of mammary rats' tumour tissues. All the slides were deparaffinised and dehydrated. For antigen retrieval, the tissue sections were pre-treated with incubation in 10mM citrate buffer (DAKO), pH 6.0 using microwave oven heat for 20 minutes. Non-specific staining reaction were blocked with peroxidase blocking reagent for 10 minutes. Incubation steps for primary antibody, secondary antibody, Streptavidin-HRP, and chromogen reagents followed protocols recommended by the manufacturer. The slides were counterstained with haematoxylin, then dehydrated and mounted. To assess the specificity of the reaction, recommended positive controls (human tonsil) and negative controls (incubation without primary antibody) were included.

Microvessel density (MVD) evaluation

The staining pattern was assessed using a light microscope according to brown DAB chromogen reaction uptake. Under X100 magnification, the pattern was reviewed in two areas: the peritumoural and intra tumoural regions. Peritumoural vessels were defined as vessels existing outside and line within or in direct contact with 50µm border surrounding the tumour while intra tumoural vessels were defined as vessels within the tumour (McGinley et al., 2002). For each region, three areas of 'hotspots' (the area with high density of microvessel profiles) were acquired by scanning the entire section under X100 magnification. Each hotspot was reviewed at X200 magnification. The positive of stained microvessels within the applied circular microscopic field were counted manually. The average of MVD was taken from the three hot-spots counts. The final MVD was the mean of the vessel counts obtained from x200 optical field. The median values of the MVD recorded were used as cut-off points to define two scores of tumours with low and high angiogenic activity.

Real Time-PCR

Total RNA was harvested from fresh tumour cells using Total RNeasy Mini Kit (Qiagen) and reverse transcribed using the iScript DNA synthesis kit. Quantitative RT-PCR assays were performed using standard Sybr Green protocol. The primer sequences of ER, PR, EGFR, ERBB2, E-cadherin and LR were listed in Table 1. For RT-PCR analysis, the exponential route of CT value were converted into a linear form using the $2^{-\Delta\Delta Ct}$ relative calculation methods. The GAPDH was used as the relative control.

Statistical analysis

Statistical analysis was performed using statistical software GraphPad Prism 7.0 for Windows. Kruskal-Wallis test with Dunn's multiple comparisons test were used to determine statistical differences of the mRNA expression of ER, PR, EGFR, ERBB2, E-cadherin and LR in control, bFGF and PF4 group. The Mann-Whitney test was chosen to compare MVD scores and gene expression data between the groups. For all statistical analysis, p values <0.05, <0.01 and <0.001 were considered as statistically significant and were marked as *, ** and ***, respectively.

Results

Histological variants of MNU-induced breast tumours subjected to bFGF-angiogenic promotion and PF4-angiogenic inhibition

Cribriform (10 out of 21 lesions) and papillary type (11 out of 21 lesions) made up most of the cases in the control group (Table 1). In the bFGF group, 11 out of 17 lesions were predominantly observed as the cribriform type (Table 1, Figure 2A). As the tumour grew, many of the cribriform type showed comedo-type changes (Figure 2A). In addition, tumour necrosis was observed, but our histological findings noted the necrosis in this group was comparable to the control group (Figure 2A). As compared

Table 1. The Distribution of Mammary Tumour Types in Control, bFGF and PF4 Groups

Tumour type	Group		
	Control	bFGF	PF4
1. Cribriform carcinoma	10	11	7
2. Papillary carcinoma	11	5	15
3. IDC-NST	-	1	3
4. Adenoid cystic carcinoma	-	-	1
Total	21	17	25

IDC, infiltrating ductal carcinoma NST, no special type

Table 2. The MVD Scoring of Control, bFGF and PF4 groups

Intervention groups	MVD SCORING			
	Peritumoural		Intratumoural	
	Low n (%)	High n (%)	Low n (%)	High n (%)
1. Normal saline (n=21)	10 (48)	11 (52)	8 (38)	13 (62)
2. bFGF (n=17)	3 (18)	14 (82)	5 (30)	12 (70)
3. PF4 (n=25)	20 (80)	5 (20)	17 (68)	8 (32)

to the control group, reduction size of tumours treated with PF4 was seen after the intervention phase. Papillary type made up most of the cases (15 out of 25 cases) in the PF4-treated group (Table 1). Our findings also noted 3 cases of the predominantly diffuse IDC-NST type in the PF4-treated group (Figure 2B), which the number is seen higher as compared to the other groups (Table 1). We also observed that tumour necrosis in this group increased compared to the control group (Figure 2C). Of all cases, no lymph nodes metastasis was observed in our model.

MVD profiles of control, bFGF-treated and PF4-treated groups

Figure 3 shows the endothelial cells that were positively stained with FVIII-RA in peritumoural and intratumoural regions. Increased number of high peritumoural MVDs (11/21, 52%) and high intratumoural MVDs (12/21, 62%) were observed in the control group (Table 2). Similarly,

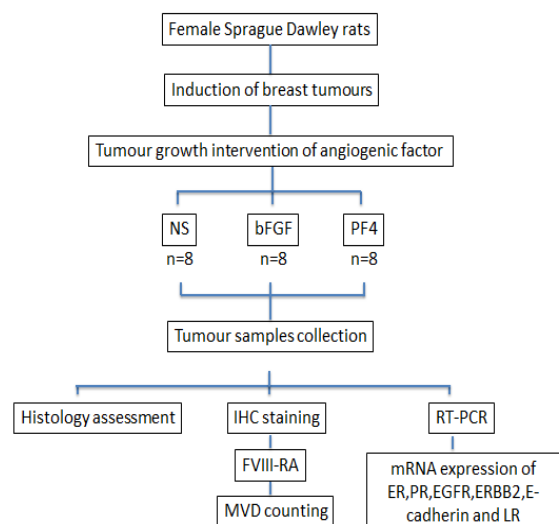


Figure 1. A Schematic Flowchart of the Study

Table 3. Association between ER, PR, EGFR, ERBB2, E-Cadherin and LR Gene Expression with Peritumoural- and Intratumoural-MVD in bFGF Group (n=17)

Gene expression		MVD SCORING					
		Peritumoural		Intratumoural			
		Low (n=3)	High (n=14)	Low (n=5)	High (n=12)		
1. ER	Median	0.04	1.192	0.12	1.978		
	p-value			**0.0059			**0.0039
2. PR	Median	1.783	2.143	1.823	2.164		
	p-value			**0.0029			* 0.0194
3. EGFR	Median	3.954	4.94	3.908	4.012		
	p-value			0.8412			0.6301
4. ERBB2	Median	4.69	4.851	4.69	4.851		
	p-value			0.7676			0.6461
5. E-CADHERIN	Median	1.749	0.7911	1.738	0.7911		
	p-value			0.0676			0.16
6. LAMININ	Median	0.12	1.812	0.271	1.912		
	p-value			0.5912			0.2786

*p<0.05, **p<0.01

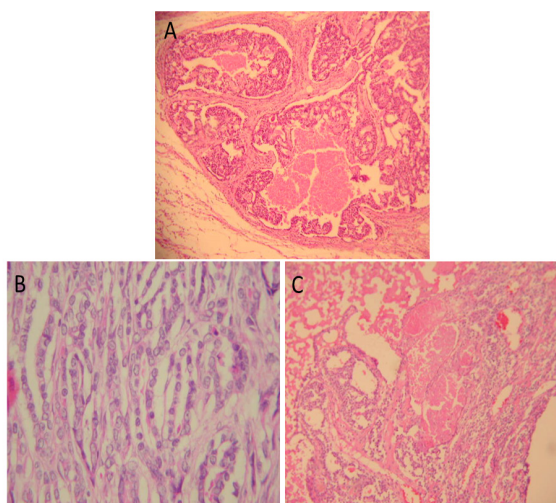


Figure 2. Histological Features of MNU-Induced Breast Tumour Subjected to Promotion and Suppression of Angiogenesis. Intervention of angiogenic factor (bFGF) and anti-angiogenic factor (PF4) were performed via intralesional injection to MNU-induced breast tumours at dose of 10µg/tumour. Histopathological assessment of the excised breast tumours was done via H&E staining as previously described (Jaafar et al., 2009). (A) A histological feature of cribriform type of bFGF-treated breast tumour at tumour size 1.6 cm (100x). While some tumour necrosis was observed, the necrosis in this group was comparable to the control group. (B) The IDC- NST type (200x) (B) Tumour necrosis (100x) observed in the PF4 group.

those breast lesions who received bFGF intervention also had greater high peritumoural MVDs (14/17, 82%) and high intratumoural MVDs (12/17, 70%) (Table 2). However, as compared to the other groups, PF4 reduces angiogenic activity as we evident increases of low MVDs at both peritumoural (20/25, 80%) and intratumoural regions (68%, 17/25) (Table 2).

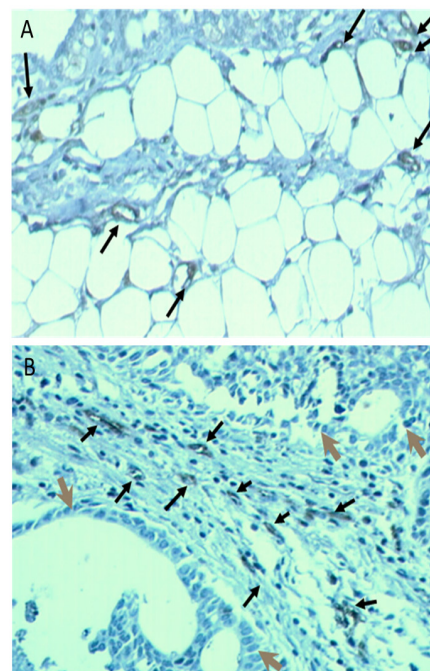


Figure 3. Positive Vascular Endothelial Cells Stained with FVIII-RA. (A) The microvessels in peritumoural region (x100). Black arrows indicate the positive vascular endothelial cells. (B) The microvessels in intratumoural region in between malignant glands (x100). Black arrows indicate positive vascular endothelial cells stained with FVIII-RA, grey arrows designate tumour cell areas.

The expression of breast tumour markers in MNU-induced breast tumours subjected to angiogenic manipulation

A real-time PCR analysis showed that promoting angiogenesis by bFGF significantly enhances ER (p<0.01), PR (p<0.001), EGFR (p<0.001), ERBB2 (p<0.001) gene expressions as compared to control group (Figure 4A-D). On the other hand, angiogenic suppression by PF4 induced ER (p<0.001) and PR (p<0.001) mRNA downregulation, which was statistically

Table 4. Association between ER, PR, EGFR, ERBB2, E-Cadherin and LR Gene Expression with Peritumoural- and Intratumoural-MVD in PF4 Group (n=25)

Gene expression		MVD SCORING			
		Peritumoural		Intratumoural	
		Low (n=20)	High (n=5)	Low (n=17)	High (n=8)
1. ER	Median	0.68	0.534	0.684	0.55
	<i>p-value</i>			*0.0102	**0.0036
2. PR	Median	0.7225	0.523	0.723	0.527
	<i>p-value</i>			**0.0010	**0.0086
3. EGFR	Median	3.965	4.995	3.969	4.511
	<i>p-value</i>			0.3356	0.7538
4. ERBB2	Median	4.335	4.946	4.492	4.85
	<i>p-value</i>			0.1004	0.5582
5. E-CADHERIN	Median	0.7666	0.7489	0.7658	0.7489
	<i>p-value</i>			0.7173	0.7007
6. LAMININ	Median	1.273	3.432	1.273	2.178
	<i>p-value</i>			0.0984	0.1808

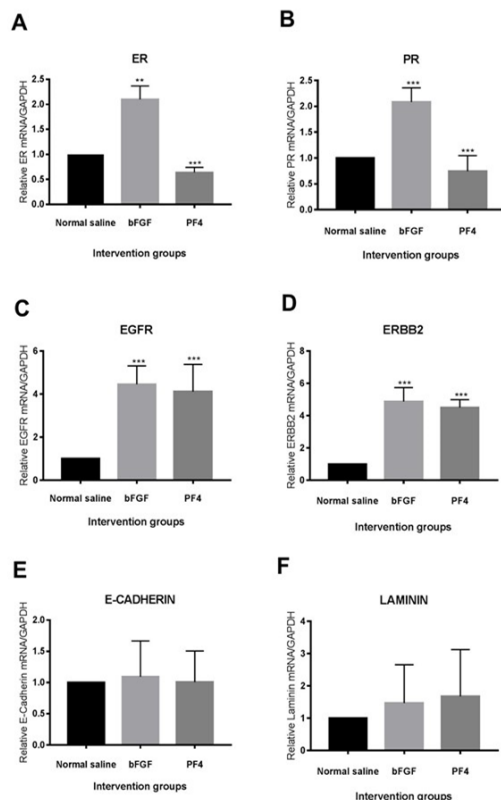
* $p < 0.05$, ** $p < 0.01$ 

Figure 4. mRNA Expressions of ER, PR, EGFR, ERBB2, E-Cadherin and LR in bFGF- and PF4-Administered Groups Compared to the Control Group. The rats were exposed to MNU carcinogen (70mg/kg of body weight) to induce breast tumours. Subsequently, tumour growth was manipulated with angiogenic factor i.e. bFGF and anti-angiogenic factor i.e. PF4, before tumours excision for RT-PCR analysis. The mRNA level (mean \pm SD) of (A)ER, (B)PR, (C)EGFR, (D)ERBB2, (E)E-cadherin and (F)LR of the treated tumours were quantified relative to the untreated tumours, and normalised to GAPDH. A Kruskal-Wallis test with Dunn's multiple comparisons test was applied to determine significant differences of receptors' expressions between groups (** $p < 0.01$, *** $p < 0.001$).

significant compared to the control (Figure 4A and B). However, EGFR and ERBB2 mRNA were seen to be significantly upregulated in tumours administered with PF4 compared to the control group (EGFR: $p < 0.001$; ERBB2; $p < 0.001$) (Figure 4C and D). Both bFGF and PF4 interventions do not influence E-cadherin and LR gene expressions, since there was no significant difference between E-cadherin and LR mRNA levels in interventions tumours (E-cadherin/bFGF: $p = 0.4352$; E-cadherin/PF4: $p = 0.1108$; LR/bFGF: $p = 0.2109$; LR/PF4: $p = 0.0715$) with compared to that in control group (Figure 4E-F).

Association between breast tumour markers expression and microvessel

We hypothesized that the degree of peritumoural and intratumoural MVD of tumour induced by bFGF and PF4 may affect the regulation of ER, PR, EGFR, ERBB2, E-cadherin and LR gene expressions. Therefore, the association of the intratumoural and peritumoural vascular density of the induced tumours with the above receptors' expressions is expected to occur in the intervention groups. In the bFGF group, relative mRNA expression of ER and PR were expressed significantly higher in tumours that scored with high MVD compared to low-MVD tumours in both peritumoural (median ER mRNA: 0.040 vs 1.192, $p < 0.01$; median PR mRNA: 1.783 vs 2.143, $p < 0.01$) and intratumoural regions (median ER mRNA expression: 0.120 vs 1.978, $p < 0.01$; median PR mRNA expression: 1.823 vs 2.164, $p < 0.05$) (Table 3). In addition, Table 4 demonstrates that ER and PR mRNA expressions were significantly reduced after intervention with PF4 in peritumoural high MVDs compared to peritumoural low MVDs (median ER mRNA expression: 0.68 vs 0.534, $p < 0.05$; median PR mRNA expression: 0.7225 vs 0.523, $p < 0.01$) and in intratumoural high MVDs compared to intratumoural low MVDs (median ER mRNA expression: 0.684 vs 0.55, $p < 0.01$; median PR mRNA expression: 0.723 vs 0.527, $p < 0.01$). However, no significant differences were found between EGFR, ERBB2,

E-cadherin and LR mRNA expressions with peritumoural and intratumoural MVDs in tumours administered with bFGF and PF4 (Table 3 and 4).

Discussion

The importance of angiogenesis in human breast cancer development has been previously described (Folkman, 1994). In this study, we determined whether changes in the cellular and molecular events of breast tumour growth are dependent or not on angiogenic activity. We first investigated the histological phenotypes of MNU-induced breast tumours subjected to promotion and suppression of angiogenesis. Cribriform subtypes and necrotic areas were observed in tumours associated with pro-angiogenic factor, bFGF but these histological findings were comparable with the finding in the control group. Overall, no critical morphological comparisons were remarked between bFGF-associated and control groups. Anti-angiogenic factor, PF4 causes a reduction in breast tumour size as well as increase incidence of tumour necrosis (Figure 2C) in our model, which is in agreement with the findings from previous studies (Giavazzi et al., 2003a; Yamaguchi et al., 2005). In addition, we observed more cases of papillary type and the appearance of diffuse NST type in the PF4-treated group (Table 1, Figure 2B). Taken together, our study suggests that inhibition of angiogenesis appears to induce reduction in differentiation of the surviving tumour cells, causing tumour aggressiveness.

Assessing MVD in extratumoural and intratumoural regions may be useful in the investigation of angiogenic manipulation in breast tumour development. In this study, the effect of pro- and anti-angiogenic factors has been shown causes significant increase and reduction in peritumoural and intratumoural MVD counts, respectively (Table 2). We further examined the expression of molecular breast tumour markers following angiogenic manipulation. A real-time PCR analysis showed that promoting angiogenesis by bFGF significantly enhances ER and PR gene expressions, which supports a previous finding that have linked high bFGF-angiogenic activity with high ER expression (Smith et al., 1999). Interestingly, it was seen that PF4 did not only suppressed angiogenic activity, but at the same time significantly downregulated ER and PR mRNA expressions. ER and PR expressions have been previously shown to correlate with tumour vascular density. Data from Table 3 and 4 demonstrates that both receptors' expressions following bFGF and PF4 interventions were significantly associated with peri- and intratumoural MVD counts. Based on the above results, the degree of angiogenic activity manipulated by bFGF and PF4 interventions is thought to influence ER- and PR- regulated proliferation and differentiation of tumour cells. Limited angiogenic activity as well as poor proliferation and differentiation activities affected by PF4 may explain the aggressive characteristics of tumours treated with this factor, as observed in our histological study.

By contrast, we demonstrated that irrespective of whether we promote or inhibit angiogenesis, the expression

of EGFR and ERBB2 continue to be significantly increased as compared to control group (Figure 4C and D). Whilst a number of previous studies showed a significant correlation between EGFR and ERBB2 expressions with MVD status in human breast cancer (Weidner and Gasparini, 1994; Koukourakis et al., 2003; Blackwell et al., 2004; Carvalho et al., 2013), however, our study was not able to determine significant associations between EGFR and ERBB2 mRNA upregulations with the MVD scores following bFGF and PF4 interventions (Table 3 and 4). In addition, there is no significant difference of E-cadherin and LR gene expressions between intervention groups as compared to that in control group (Figure 4E and F).

We concluded that only ER and PR, two well-known prognostic and predictive markers for invasive breast carcinomas (Russo and Russo, 2006), show a consistent response when tumour angiogenesis were manipulated either positively or negatively. Our study follows with current understanding that not only do we need to target hormonal receptors, as presently practiced, but we also need to target endothelial receptors to successfully treat breast cancer. To date, selective ER modulators (SERM) e.g. tamoxifen which have been used to treat ER-positive breast cancers also appear to have antiangiogenic potency (Hurteau et al., 2010; Mele et al., 2010). However, the underlying molecular mechanism by which tamoxifen inhibits angiogenesis remains limited. As we found that anti-angiogenic causes anti ER/PR action via PF4, we believe that by integrating PF4 activity could be one of the most important strategies to produce VEGF/FGF-related estrogen antagonist. Recent study has shown that a combination treatment of PF4 and rapamycin (mTOR inhibitor) up-regulates pro-apoptotic programmed cell death in tumour cells that are unable to undergo Bax/Bcl-2-mediated apoptotic cell death (Al-Astani Tengku Din et al., 2014). For this, further work must be done to develop such a drug. Further studies should be designed to identify possible potential endothelial-associated antigen/protein that can be targeted for treatment, as well as for use as a diagnostic tool in predicting aggressiveness of breast cancer.

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