Expression of endothelial PDGF receptors α and ß in breast cancer: Up-regulation of endothelial PDGF receptor ß

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Abstract. The PDGF pathway is essential in tumor angiogenesis. Although the expression of the PDGF receptors has been excessively studied on breast cancer cells, few studies exist on PDGFR expression on the tumor endothelial cells. In the present study, it is investigated whether endothelial PDGF receptors' expression is altered in breast cancer. Endothelial PDGFRα and ß expression was initially studied under the influence of tumor conditioned medium derived from a breast cancer cell line. Following tissue culture experiments the endothelial expression of both receptors was studied on formalin-fixed paraffin-embedded tissue sections of normal breast and breast cancer specimens. The tissue culture experiment revealed a possible up-regulation of endothelial PDGFRß by breast cancer environment. Immunohistochemistry verified the result since 69.7% of the breast cancer sections were positive for PDGFRß compared to 43.3% of normal breast sections ($p<0.05$). No statistical difference was revealed by studying PDGFRα expression. In conclusion, our findings support the thesis of possible anti-PDGFRß anti-angiogenic therapy, in cases of endothelial PDGFRß-expressing breast cancer.

Introduction

In order for a primary tumor to exceed a certain size, the establishment of a vascular network is a clear necessity (1). This network is formed as the result of the tumor effect upon adjacent (2) or circulating endothelial cells (3). Under the influence of a tumor-derived microenvironment, endothelial cells proliferate and organize to neo-capillaries, supplying the tumor site with nutrients and oxygen. Furthermore, these capillaries offer a direct route, via the bloodstream, for the primary tumor to disseminate (4).

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Angiogenesis has been studied in many different tumor models and its role in cancer progression is well established (4). However, the theory of the 'angiogenic switch' supports the thesis that the net result as far as angiogenesis is concerned, depends on the balance between angiogenic and antiangiogenic factors (5). Taking under consideration the fact that neoplasms behave differently, referring to the factors that they may secrete, it becomes clear that endothelial cells involved in angiogenesis, respond to a message that possibly features the primary tumor site and histotypic origin.

In breast cancer, several factors have been identified as promoters or inhibitors of angiogenesis, most common of which are the VEGF family, the FGF family, the PDGF family and the relevant receptors (6). Many studies have been performed correlating these factors and their receptors with clinical features such as tumor grade and overall survival. Among these factors and their receptors, the PDGF/PDGFR pathway has become of interest, especially due to the existence of the commercially available inhibitor imatinib (7). Although this pathway is well-studied in breast cancer cells (8,9), only a single report exists describing PDGFR expression in breast cancer endothelial cells (10).

We studied the expression of PDGF receptors α and ß on endothelial cells grown in a breast cancer environment. Moreover, using archival material we have studied whether these findings are verified in human breast cancer tissues. The main aim was to prove a possible PDGFR up-regulation, a fact that would provide evidence for further evaluation of the role of imatinib and other PDGFR inhibitors, in breast cancer.

Materials and methods

Cell lines. The EA.hy 926 endothelial cell line (11) was grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose concentration (4500 mg/l) supplemented with gluttamax-I, 10% fetal bovine serum (FBS) and penicillin/streptomycin. The MDA MB-468 breast cancer cell line was cultured in DMEM with low glucose concentration (1500 mg/l) supplemented with gluttamax-I, 10% FBS and penicillin/streptomycin. Both cell lines were maintained in a 5% CO_2 atmosphere, in a humidified incubator. All tissue culture media were purchased from Invitrogen, Scotland, UK and the tissue culture plasticware were from Costar, USA.

Table I. Main features of the antibodies used in this study.

Antibody/source	Antigen retrieval	Dilution	Incubation time	Detection system
CD31, Dako, Denmark	MW[a]	1/25	1 h	Super Sensitive™ link-label detection system, Biogenex, USA
PDGFRα, Neomarkers, USA	Saponine[b]	1/40	ON	Anti-polyvalent ultra vision detection system, Lab Vision, USA
PDGFRß, Santa Cruz, USA	Saponine[b]	1/40	ON	Same as PDGFRα

[a]MW (350W for 5 min) in 0.01 M citrate buffer, pH 6.2, incubation 30 min in room temperature and repetition. [b]Saponine (50 mg/100 ml dH_2O) for 30 min in room temperature, followed by washing with dH_2O.

Tumor conditioned medium (TCM) preparation. The MDA MB 468 cell line was cultured in the medium mentioned above. After confluence had been reached, the cells were washed with phosphate buffer saline (PBS) and the medium was replaced with FBS-free DMEM with a high glucose concentration. Culture was maintained for 48 h. The TCM was then collected, cleared by centrifugation and stored in aliquots at -80˚C. Each aliquot was thawed once prior to use.

EA.hy 926 endothelial cell line culture in tumor environment. The EA.hy 926 endothelial cells were grown on negatively charged SuperFrost Plus slides as previously described (12). After confluence had been reached the culture medium was removed, followed by a PBS wash. Then the cells were left to grow in TCM for 24 h. Endothelial cells grown in FBS-free medium served as controls. The slides were collected and the cells were fixed in 4% paraformaldehyde in PBS for 10 min. Slides were stored immersed in 70% ethanol, until immunostained.

Patients. Thirty-three cases of breast cancer and 30 cases of normal breast tissue were randomly chosen from the archives of the Laboratory of Pathology of the University Hospital of Heraklion, Crete, Greece. All tissues had been fixed in 4% buffered formalin and embedded in paraffin according to the normal schedule used in the laboratory. From each block, 3-μm-thick sections were cut, mounted on SuperFrost Plus slides and dried at 50˚C for 1 h. The sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water.

Immunocytochemistry and immunohistochemistry. Both cell and tissue samples were processed in one batch in order to avoid differences in staining due to the technique. Following antigen retrieval, the Ea.hy 926 cells were stained for PDGFRα and PDGFRß, while tissue samples were stained as well for CD31. Immunodetection of the PDGF receptors was performed using the anti-polyvalent ultra vision detection system (Lab Vision, Fremont, CA, USA), while CD31 was detected by using the Super Sensitive™ link-label IHC detection system (BioGenex, San Ramon, CA, USA), according to the manufacturers' protocols. Details regarding antigen retrieval and primary antibodies are presented in Table I. Finally, the slides were washed with deionized water, counterstained for 30 sec with Papanicolaou 1a Harris hematoxylin (Merck KGaA 64271, Germany), and cover-slipped with glycergel (USA).

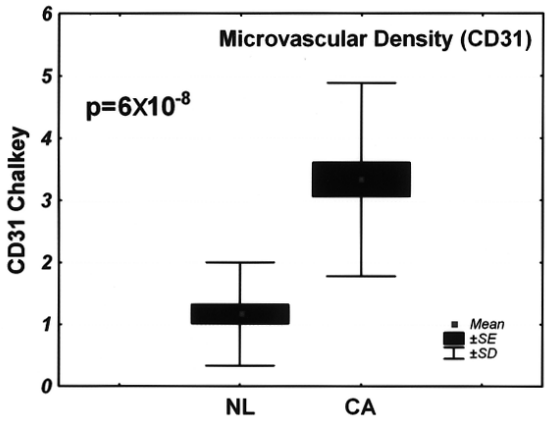

Figure 1. Microvascular density distribution among breast cancer and normal breast specimens. It is clear that angiogenic activity is increased in breast cancer.

Microvascular density (MVD). Microvascular density was measured as previously described (13). Briefly, the Chalkey count was applied by two independent observers, counting as vessel any CD31 positively-stained endothelial cell. Counting was performed on a hot-spot recognized by consensus.

Statistical analysis. For analyzing differences in expression between normal and tumor tissue samples, the χ^2 test was applied. In order to discriminate significant differences in microvascular density between tissues expressing PDGF receptors and tissues that do not express PDGF receptors, the Mann Whitney test was applied. An observation with $p<0.05$ was considered statistically significant.

Results

Breast cancer is featured by increased angiogenic rate. The effect of the TCM from the MDA MB-468 cell line on the EA.hy 926 endothelial cells has been previously reported by our group (14). For angiogenesis to be quantified in paraffin-embedded tissue sections, the well-accepted Chalkey count method was applied. As shown in Fig. 1 the mean micro-

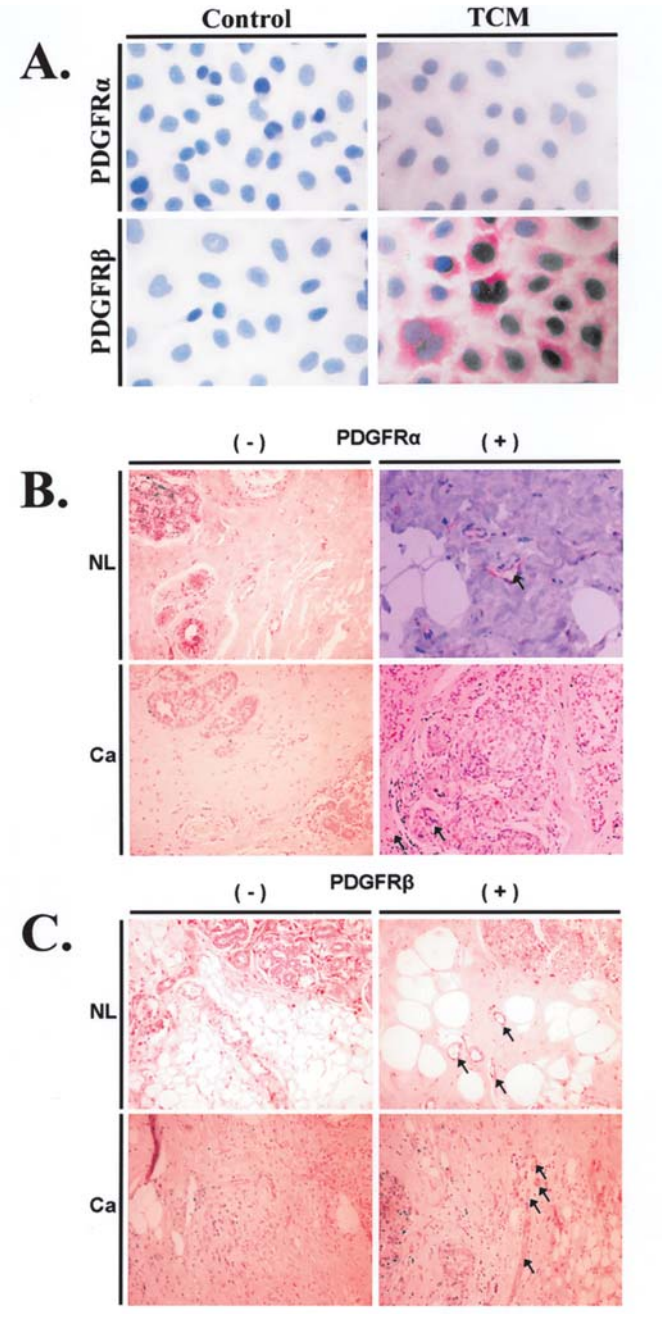

Figure 2. Immunocytochemistry results of PDGFRα and PDGFRß staining, on the EA.hy 926 endothelial cells grown in the presence or in the absence of tumor conditioned medium (A). Endothelial PDGFRß is up-regulated by TCM. Immunohistochemistry results among typical cases of normal breast and breast cancer specimens using anti-PDGFRα (B) and anti-PDGFRß (C). The arrows indicate positively stained endothelial cells.

vascular density value for the normal breast tissues was 1.16±0.15, while the respective value for breast cancer tissues was 3.33±0.27. The difference was significant ($p<0.05$, Mann-Whitney test).

Breast cancer cells induce PDGFRß but not PDGFRα expression in the adjacent endothelial cells. As shown in Fig. 2 when the EA.hy 926 endothelial cells are grown in TCM from the MDA MB 468 breast cancer cell line, they exhibit positive reactivity for PDGFRß. On the contrary, no reactivity is detected for PDGFRα. Immunohistochemistry for both endothelial PDGF receptors revealed a significant difference ($\chi^2=4.45$, $p=0.03$) between normal breast and breast cancer tissue samples with positive PDGFRß staining (Table II). On the other hand, nearly all samples expressed PDGFRα and thus, no significant difference was found between normal breast and breast cancer tissue samples (Yates $\chi^2=1.33$, $p=0.24$) (Table II).

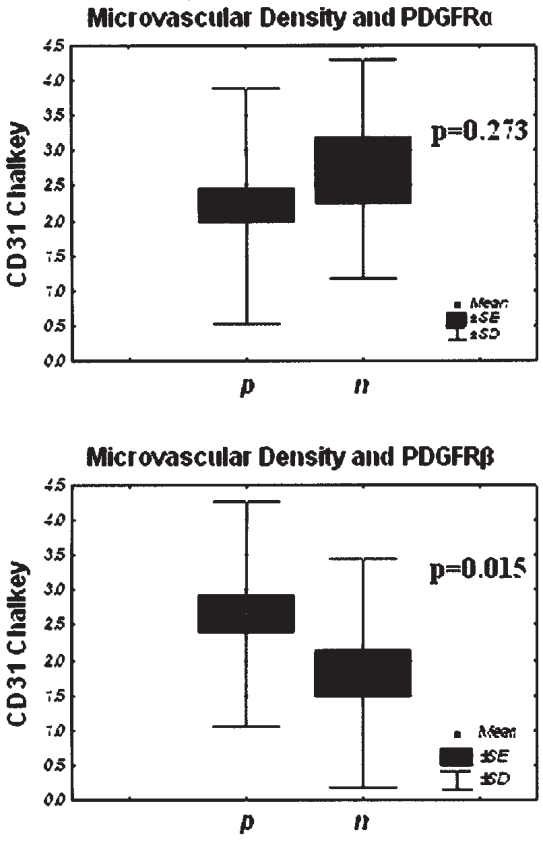

Figure 3. Microvascular density distribution among endothelial PDGF receptor-expressing *(p)* and PDGF receptor non-expressing tissues *(n)*. PDGFRß-expressing tissues are presented with higher angiogenic activity (Mann Whitney U = 312.00, p=0.015). No significant MVD difference was found between PDGFRα-expressing and non-expressing breast tissues (Mann Whitney U = 225.00, p=0.273).

In order to examine which receptor is related to angiogenesis in breast tissues, the specimens where categorized based on the endothelial expression or not of each receptor. The breast tissues (Fig. 3) expressing PDGFRß present a significantly increased (Mann-Whitney test, $p<0.05$) angiogenic activity (mean MVD value: 2.66±0.26), compared to PDGFRß non-expressing breast tissues (mean MVD value: 1.81±0.31). On the contrary, no significant difference (Mann-Whitney test, $p>0.05$) was revealed by comparing PDGFRα expressing breast tissues (mean MVD value: 2.21±0.23) and PDGFRα non-expressing breast tissues (mean MVD value: 2.72±0.46).

Discussion

Tumor angiogenesis is an essential process for a primary tumor to grow and disseminate. Angiogenesis is achieved via signal transduction through many different pathways, one of

Table II. Results of the immunohistochemical study probing endothelial PDGFRα and PDGFRß.[a]

	Endothelial PDGFRα (+)	Endothelial PDGFRα (-)	Endothelial PDGFRß (+)	Endothelial PDGFRß (-)
Normal breast samples (n=30)	27 (90.00%)	3 (10.00%)	13 (43.33%)	17 (56.67%)
Breast cancer samples (n=33)	25 (75.76%)	8 (24.24%)	23 (69.70%)	10 (30.30%)
P (after proper χ^2 test)	0.24 (>0.05)		0.03 (<0.05)	

[a]There is a significant difference in the expression of endothelial PDGFRß between breast cancer and normal tissue samples.

which is the PDGF/PDGFR pathway (15). PDGF receptors have been described to play a critical role in tumor progression as part of the group of receptors expressed on the membrane of tumor cells (16). Especially in breast cancer, PDGF receptors are recognized in cases of carcinomas with aggressive behaviour (17). Although well studied in breast cancer tumor cells, the PDGFR expression has not been extensively studied on the tumor stroma-associated endothelial cells. It is reported that PDGF receptors are expressed in stromatic cells in cases of both normal or cancer tissue (10), without referring to the endothelial cells that are activated to form new capillaries. A more recent approach describes PDGFRß to be expressed by endothelial cells grown in a bone metastasis breast cancer model (18). The present study is the first to describe endothelial PDGFRß up-regulation, as a consequence of breast cancer cell induction. This finding could be of possible future clinical importance, especially nowadays that several tyrosine kinase inhibitors are tested as inhibitors of both tumor progression and/or tumor angiogenesis.

Additionally, the tissue culture experiment demonstrated that PDGFRα up-regulation was unlikely to happen due to breast cancer cell induction, since neither in the presence nor in the absence of TCM did the EA.hy 926 endothelial cells express PDGFRα. However, the immunohistochemical results where not absolutely consistent with the tissue culture experiment, since nearly all samples expressed PDGFRα. This disagreement could be explained by the fact that the *in vitro* experiment represents merely a single case. Thus, the fact that the EA.hy 926 cells did not express PDGFRα may represent one of the rare cases occurred in the immunohistochemical study.

Endothelial PDGFRß expression has not been correlated so far with any clinical feature of breast cancer. Although PDGFRß is reported to be expressed by tumor vasculature in breast cancer metastasis to bone tissue, there is no study investigating endothelial PDGFRß expression to the primary site. Moreover, no correlation with progression of the disease or the overall survival has been made. Our study is the first to correlate endothelial PDGFRß with increased angiogenesis in breast tissues (Fig. 3). Nevertheless, the number of samples is small and needs expansion to extrapolate the findings to the general population.

The quantification of the angiogenic activity was performed by applying the Chalkey count method. This method is considered one of the most reliable in calculating an approximation of a cellular population among a microscopic field of view, and it is based on a random surface distribution. Therefore, when applied on a hot-spot, the Chalkey count succeeds in calculating in a reproducible way the ratio of the positively stained cells.

Our finding could be of importance if PDGFRß was used as a target of molecular interventions, with the aim of inhibiting breast cancer angiogenesis and therefore disease progression (19). It has been shown by our group and others, that endothelial cell growth rate may be reduced or even inhibited in the presence of imatinib, a well-studied PDGFR inhibitor (20). By selecting patients whose primary tumor expresses endothelial PDGFRß, angiogenesis inhibition could possibly be achieved, ensuring at least down-staging of the disease.

The verification of imatinib or other anti-PDGFRß agents as efficient angiogenic inhibitors, demands a properly designed trial, taking under consideration PDGFRß expression. Although imatinib is reported to succeed in second level clinical trials as a single-agent therapeutic regimen against metastatic breast cancer (21), this discrimination of cases to endothelial PDGFRß positive or negative has not been described so far. It is anticipated that the therapeutic outcome of inhibiting PDGFRß with imatinib (or other inhibitors), would be maximized in the cases that express PDGFRß.

Finally, the mechanism by which PDGFRß is up-regulated in tumor vasculature is to be studied in the future. Recent data support the thesis that FGF-2 up-regulates PDGFRß expression in a murine model, thus enhancing angiogenesis (22). Moreover, hypoxia is reported to trigger PDGFRß expression in the context of activating pathways that will eventually lead to expression of survival/antiapoptotic genes (23). Both observations may hold also in breast cancer, since members of the FGF superfamily are definitely secreted by breast cancer cells (24) and intra-tumor hypoxia is a proven condition in breast cancer (25). Experiments towards this direction may involve FGF neutralizing antibodies in endothelial cell culture and immunohistochemistry staining regarding expression of FGF on breast cancer cells. Correlation between endothelial PDGFRß and HIF-1α staining could also be studied to verify a connection between hypoxia and endothelial PDGFRß expression.

In conclusion, we have demonstrated that the tumor endothelial cells in breast cancer may express PDGFRß, possibly via induction by breast cancer cells. This finding may support further studies in proving PDGFRß inhibitors useful as chemotherapeutic agents against breast cancer.

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References

1. Folkman J: Role of angiogenesis in tumor growth and metastasis. Semin Oncol 29 (Suppl 16): 15-18, 2002.
2. Hanahan D and Weinberg RA: The hallmarks of cancer. Cell 100: 57-70, 2000.
3. Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G and Bertolini F: Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. Blood 97: 3658-3661, 2001.
4. Papetti M and Herman IM: Mechanisms of normal and tumor-derived angiogenesis. Am J Physiol Cell Physiol 282: C947-C970, 2002.
5. Hanahan D and Folkman J: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86: 353-364, 1996.
6. Rice A and Quinn CM: Angiogenesis, thrombospondin, and ductal carcinoma *in situ* of the breast. J Clin Pathol 55: 569-574, 2002.
7. Buchdunger E, O'Reilly T and Wood J: Pharmacology of imatinib (STI571). Eur J Cancer 38 (Suppl 5): 28-36, 2002.
8. Bronzert DA, Pantazis P, Antoniades HN, Kasid A, Davidson N, Dickson RB and Lippman ME: Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. Proc Natl Acad Sci USA 84: 5763-5767, 1987.
9. Coltrera MD, Wang J, Porter PL and Gown AM: Expression of platelet-derived growth factor B-chain and the platelet-derived growth factor receptor beta subunit in human breast tissue and breast carcinoma. Cancer Res 55: 2703-2708, 1995.
10. Bhardwaj B, Klassen J, Cossette N, Sterns E, Tuck A, Deeley R, Sengupta S and Elliott B: Localization of platelet-derived growth factor beta receptor expression in the periepithelial stroma of human breast carcinoma. Clin Cancer Res 2: 773-782, 1996.
11. Edgell CJ, McDonald CC and Graham JB: Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci USA 80: 3734-3737, 1983.
12. Vrekoussis T, Stathopoulos EN, Kafousi M, Darivianaki A and Zoras O: Establishment of a new co-culture model using negatively-charged slides. In Vivo 18: 471-476, 2004.
13. Vermeulen PB, Gasparini G, Fox SB, Colpaert C, Marson LP, Gion M, Belien JA, de Waal RM, Van Marck E, Magnani E, Weidner N, Harris AL and Dirix LY: Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. Eur J Cancer 38: 1564-1579, 2002.
14. Vrekoussis T, Stathopoulos EN, Kafousi M, Saridaki Z, Sanidas E and Zoras O: Endothelial p21(WAF1/CiP1) cell cycle inhibitor is down-regulated in breast cancer. Anticancer Res 25: 2743-2748, 2005.
15. Ostman A: PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. Cytokine Growth Factor Rev 15: 275-286, 2004.
16. Yu J, Ustach C and Kim HR: Platelet-derived growth factor signaling and human cancer. J Biochem Mol Biol 36: 49-59, 2003.
17. Carvalho I, Milanezi F, Martins A, Reis RM and Schmitt F: Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression. Breast Cancer Res 7: R788-R795, 2005.
18. Lev DC, Kim SJ, Onn A, Stone V, Nam DH, Yazici S, Fidler IJ and Price JE: Inhibition of platelet-derived growth factor receptor signaling restricts the growth of human breast cancer in the bone of nude mice. Clin Cancer Res 11: 306-314, 2005.
19. Board R and Jayson GC: Platelet-derived growth factor receptor (PDGFR): a target for anticancer therapeutics. Drug Resist Updat 8: 75-83, 2005.
20. Vrekoussis T, Stathopoulos EN, De Giorgi U, Kafousi M, Pavlaki K, Kalogeraki A, Chrysos E, Fiorentini G and Zoras O: Modulation of vascular endothelium by imatinib: a study on the EA.hy 926 endothelial cell line. J Chemother 18: 56-65, 2006.
21. Modi S, Seidman AD, Dickler M, Moasser M, D'Andrea G, Moynahan ME, Menell J, Panageas KS, Tan LK, Norton L and Hudis CA: A phase II trial of imatinib mesylate monotherapy in patients with metastatic breast cancer. Breast Cancer Res Treat 90: 157-163, 2005.
22. Kano MR, Morishita Y, Iwata C, Iwasaka S, Watabe T, Ouchi Y, Miyazono K and Miyazawa K: VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFRbeta signaling. J Cell Sci 118: 3759-3768, 2005.
23. Zhang SX, Gozal D, Sachleben LR Jr, Rane M, Klein JB and Gozal E: Hypoxia induces an autocrine-paracrine survival pathway via platelet-derived growth factor (PDGF)-B/PDGF-beta receptor/phosphatidylinositol 3-kinase/Akt signaling in RN46A neuronal cells. FASEB J 17: 1709-1711, 2003.
24. Dickson C, Spencer-Dene B, Dillon C and Fantl V: Tyrosine kinase signalling in breast cancer: fibroblast growth factors and their receptors. Breast Cancer Res 2: 191-196, 2000.
25. Boudreau N and Myers C: Breast cancer-induced angiogenesis: multiple mechanisms and the role of the microenvironment. Breast Cancer Res 5: 140-146, 2003.