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PRINCIPAL INVESTIGATOR: Elly-Gerald Stoica, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington DC 20057

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14. ABSTRACT

Induction of neovascularization is needed for a growing tumor as well as for its metastasis. Angiogenic and growth promoting factors like Pleiotrophin (PTN) act on endothelial and epithelial cells and on fibroblasts. We identified the receptor for PTN as anaplastic lymphoma kinase(ALK). In individual tissues the presence of ALK is elevated in tumor stroma (endothelium) while adjacent normal tissue lacked ALK. In cultured endothelial cells or human fibroblasts ALK is upregulated in response to supernatants from human breast cancer cells. Our hypothesis is that ALK from stromal cells, upregulated in response to factors from tumor cells, constitutes a marker and a potential therapeutic target in breast cancer. We investigated the specificity of ALK modulation in tumor stroma versus normal endothelium in response to growth factors and breast cancer cell lines supernatants. We underscored the importance of the ALK/PTN axis in migration and invasion and we blocked these effects with blocking antibodies. The completion of the study will translate in establishing ALK as a new target for the breast metastatic cancer therapy.

15. SUBJECT TERMS
Breast Cancer, tumor-stroma interaction, neoangiogenesis

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Introduction

Growth factors that are secreted by the tumor cells are eliciting host responses crucial for survival, proliferation and metastasis (1). A critical event in this respect is the induction of neovascularization needed for a growing tumor as well as for its metastasis. In breast cancer, microvessel density is an independent prognostic indicator and there is a direct correlation between blood vessel density in primary tumors and their metastasis. Solid tumors cannot develop in absence of new blood vessels; therefore, the malignant cells would co-opt endothelial cells and fibroblasts to sustain their growth. The latter cells are sensitive to angiogenic and growth promoting factors like Pleiotrophin (PTN) which supports the transition from the avascular to the vascular stage. We identified the receptor for PTN as anaplastic lymphoma kinase (ALK), a formerly orphan tyrosine kinase receptor (2). Preliminary studies showed that in individual tissues the presence of ALK is elevated in tumor stroma (endothelium) while adjacent normal tissue lacked detectable ALK expression. Moreover, experiments performed in cultured endothelial cells (HUVEC) or human fibroblasts (WI-38) showed that ALK is upregulated in response to basic FGF (b-FGF) and supernatants from human breast cancer cells.

Our hypothesis is that ALK from stromal cells, upregulated in response to factors from tumor cells, constitutes a marker and a potential therapeutic target in breast cancer.

Proposal body:

In the approved statement of work the following tasks were outlined:

Task 1 Investigate the specificity of ALK up regulation in tumor stroma versus normal endothelium (months 1-6)

Task 2 Substantiate the ALK up regulation in stroma in response to growth factors and breast cancer cell lines supernatants (months 1-6)

Task 3 Demonstrate the effects of ALK up regulation at the signal transduction level. (months 2-8)

Task 4 Investigate the functional effects of the differences in ALK signaling (months 7-11)

Task 5 Detect the differences in drug sensitivity in endothelial cells that have an increased ALK level versus untreated cells. (months 10-11).

Task 6 Analyze data and prepare report for DOD (month 12)

Due to complexity of our tasks and unforeseen circumstances (medical leave of one of the participants in the project) we have requested and were granted a one year no-cost extension from DOD USAMRMC.

Task 1 Investigate the specificity of ALK up regulation in tumor stroma versus normal endothelium (months 1-6).

We completed task 1 in the months 1-5. We performed a detailed analysis of the presence of PTN in breast cancer specimens and the expression of ALK in the epithelium and stroma of breast cancer tissue arrays. Moreover, we correlated these two markers with the long term survival of the patients from which the tissues were prevailed (Figure 1). Our results indicate that there is a significant difference between the 10 year survival for patients expressing high levels of PTN versus the negative cases (~ 50 % in the Kaplan-Meier survival curve). 35% of the patients that did not express PTN survived for 20 years while only 10% of the patients with high levels of PTN survived that amount of time. A similar conclusion may be drawn for ALK expression: for high expressers the 10-year survival was ~30% while 60% of the patients in which ALK was not detected survived 10 years or longer. At 20 years, 30% of the negative for ALK cases were living while no patients who expressed high levels of ALK survived more than 160 months.

Task 2 Substantiate the ALK up regulation in stroma in response to growth factors and breast cancer cell lines supernatants (months 1-6).

a) We had great difficulties in obtaining and establishing human breast reduction primary fibroblasts. Initially, we postponed this subtask but eventually we had to abort it and focus our resources on cancer cell lines in tissue culture.
b) We collected conditioned media from the supernatants of the breast cancer cell lines MDA MB 231, Hs578 T, MCF-7, as well as other cancer lines and we used them to stimulate endothelial cells and fibroblasts.
c) Using RT PCR and Western blotting we measured the ALK levels (mRNA and protein) in HUVEC cells in response to bFGF, IGF, PDGF, EGF, Heregulin or conditioned media (obtained as above) treatment as presented in figure 2. The experiments on HMVEC, WI- 38 and another normal human lung fibroblastic cell line IMR-90 were not successful as we were not able to observe the same behavior as in HUVECs in a constant and reliable pattern. We decided to focus our attention on the HUVECs.
d) Besides bFGF, EGF, IGF-1 and PDGF, which were previously detected, we were not able to conclude which are the growth factors present in the conditioned media that would be used by the cells to upregulate ALK by using blocking antibodies. Another approach could be, instead of using blocking antibodies, a proteomic analysis of the conditioned media for various cancer cell lines that induce upregulation of ALK. However, the costs associated with such an approach are far beyond the budget for this Concept Award.

Task 3 Demonstrate the effects of ALK up regulation at the signal transduction level.

a) Monitor the activation of ALK by tyrosine phosphorylation of the receptor and by activation of downstream signaling molecules like Akt and MAPK in cellular lysates obtained as in Task 2.

As presented in figure 3, in HUVECs the ALK receptor is phosphorylated in response to PTN and the amount of phosphorylation depends on the presence of the ligand and the type of pretreatment of which the HUVECs were exposed to. Presented in figure 3 is also the FAK phosphorylation on tyrosine 397. FAK plays a central role in cell spreading, differentiation, migration, cell death and acceleration of the G1 to S phase transition of the cell cycle. Tyrosine 397 is the autophosphorylation site of FAK, and involved in its initial activation.

b) Perform dose response studies with small molecules that inhibit ALK, AKT, and MAPK phosphorylation to detect the extent to which the ALK signaling is enhanced/modified.

We started a very promising collaboration with Dr. Alan Kozikowsky from University of Chicago and we already have some lead compounds that act as inhibitors of ALK (Figure 4). The compounds were modeled after the IGF-R binding pocket and should be close to the ALK ATP binding pocket since ALK is part of the Insulin Receptor family. The next step would be to test the specificity of the inhibitors and to generate the second generation with better inhibitory efficacy

Task 4 Investigate the functional effects of the differences in ALK signaling (months 7-11)

a) Test the proliferative effects of PTN on pretreated versus not treated endothelial cells (HUVEC and HMVEC) or fibroblasts (WI-38) by WIST system. (months7-8)

We did not detect any difference in the proliferative properties of the pretreated vs. untreated cells.

b) Test the anti apoptotic effects of PTN on pretreated versus not treated endothelial cells (HUVEC and HMVEC) or fibroblasts (WI-38) by Annexin V-FITC system. (months7-8)

In our experiments, no differences in the serum withdrawal induced apoptosis rate of the treated vs. untreated cells was detected.

c) Test the potential differences in PTN induced cell migration of fibroblasts and endothelial cells by Boyden chamber assays using standard protocols from the Developmental Therapeutics Program- NCI/NIH (months 8-9).

It appears that the functional significance of PTN/ ALK axis lies in the capacity of inducing migration of HUVECs and fibroblasts as well in other cells that rely on this pathway. We were able to demonstrate the change in the migration rate of HUVECs in response to PTN after pretreatment with growth factors and cancer cells conditioned media (Figure 5) and the blocking of the effect with anti ALK mouse monoclonal antibody 8B10. Moreover fibroblasts (NIH 3T3 cells) responded in a PTN dose dependent manner in a Boyden chamber assay and the migration could be inhibited by an

ALK monoclonal antibody as well as by PI3 Kinase and Src inhibitors but not by MAP kinase inhibitors (figure 6).

The role of PTN in HUVECs migration was under scored by the dose dependent reduction of the role of barrier function of the cells in an ECIS experiment (figure 7). Briefly, the HUVECs were let to adhere to a special cuvete that contain electrodes. Once the confluence is attained, the cells will have a set value of Impedance when challenged with a high frequency electric current that does not change the integrity of the cells. If the cells are displaced from the electrode (either by an invasive cell type that will penetrate between the HUVEC monolayer or by activating migration of HUVECs) there will be a measurable effect of the Impedance change.

d) Uncover potential differences in PTN induced tube formation of endothelial cells, using standard protocols from the Developmental Therapeutics Program-NCI/NIH. (months 9-10).

The experiments performed did not detect any differences in the pretreatment of the endothelial cells in the tube formation assay. However, we also pursued another approach in vivo. We generated matrigel plugs that contained bFGF. They were implanted subcutaneously in the abdominal region of mice and then retrieved to detect neoangiogenesis. We were able to block the formation of blood vessels by using an anti ALK monoclonal antibody thus underscoring the involvement of ALK in the process (Fig.8) Moreover, the detection of ALK (by in situ hybridization) paralleled the Immunohistochemical detection of proliferating endothelial cells as detected by anti PCNA staining (Figure 9)

Task 5 Detect the differences in drug sensitivity in endothelial cells that have an increased ALK level versus untreated cells. (months 10- 11)

a) Perform dose-response growth assay (DTP-NIH) using the fumagillin derivative TNP-40, paclitaxel (Taxol) and doxorubicin on the HUVEC and HMVEC cells pretreated as in Task 2. (months 10-11)

We did not detect any notable differences in the behavior of pretreated vs. untreated endothelial cells in response to TNP-40, Taxol and doxorubicin.

Task 6 Analyze data and prepare report for DOD (month 12)

Key research accomplishments:
• Establishing a correlation between the expression of PTN and ALK in breast cancer stroma and long term survival.
• Underscore the role of tumor secreted growth factors in upregulation of ALK

• Detecting the crucial role played by the PTN/ALK axis in the migratory behavior of Huvec cells
• Establishing the connection between neoangiogenesis and ALK in vivo.

Reportable outcomes: G.Stoica, E. Bowden, A. Kuo, D. Kodack, A. Wellstein. The role of ALK in the prevention of metastasis in breast cancer. Manuscript in preparation.

Conclusion

We investigated the ALK upregulation in tumor stroma versus normal endothelium in response to growth factors and breast cancer cell lines supernatants. Also, we targeted our work towards blocking the ALK activation, either by small molecule inhibitors or blocking antibodies. The study will establish ALK as a new target for the breast cancer therapy and especially metastatic cancer.

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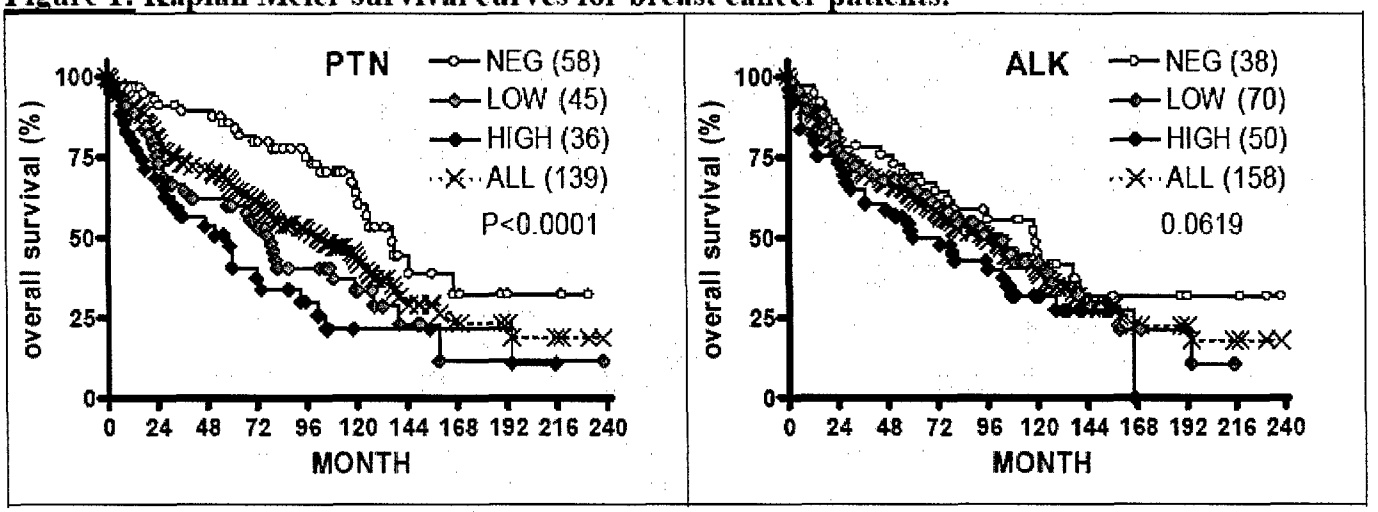
Figure 1: Kaplan Meier survival curves for breast cancer patients.



NEG, LOW, HIGH: Curves of patients with the indicated expression level, **ALL**: all tumors regardless of expression. **In brackets:** number of samples per group. **P-values**: Results of log rank test for trend Kaplan Meier analysis

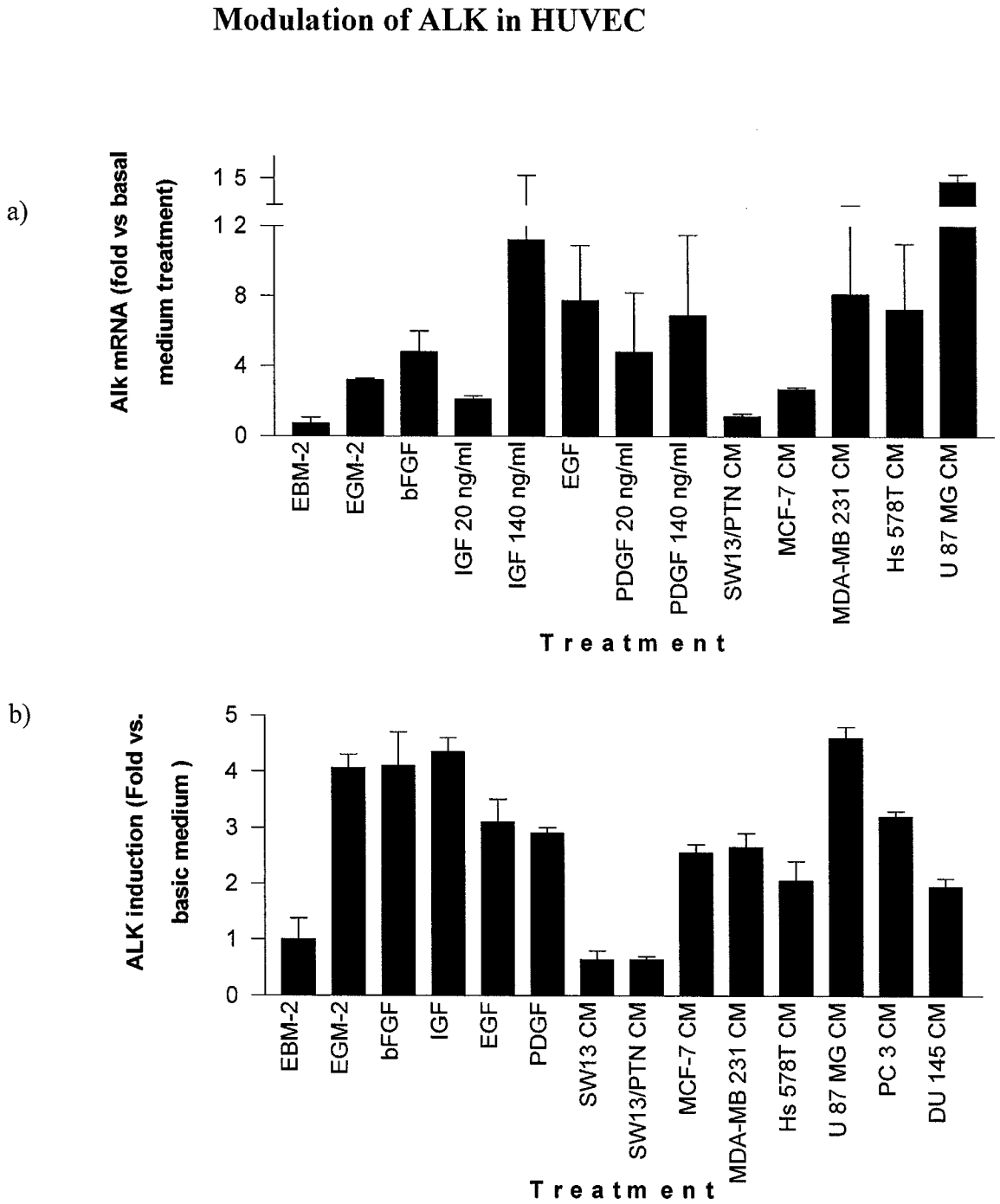


Figure 2. Upregulation of ALK in HUVEC in response to growth factors and conditioned media from various cell lines. a) The levels of ALK mRNA, as determined by RT-PCR, upon treatment with bFGF 10 ng/ml, IGF-I, PDGF, EGF 100ng/ml, and conditioned media from SW-13, MCF-7, MDA-MB-231, Hs578T, U 87 MG cell lines. EBM-2 is the basal medium and EGM-2 is the growth medium for HUVEC (Clonetics, Walkersville, MD) b) ALK modulation at the protein level after treatment of HUVEC with bFGF 10 ng/ml, IGF-I 140 ng/ml, PDGF 140 ng/ml, EGF 100ng/ml, and conditioned media from SW-13, SW-13 transfected with PTN (SW-13/PTN), MCF-7, MDA-MB-231, Hs578T, U 87 MG, PC3 and DU 145 cell lines.

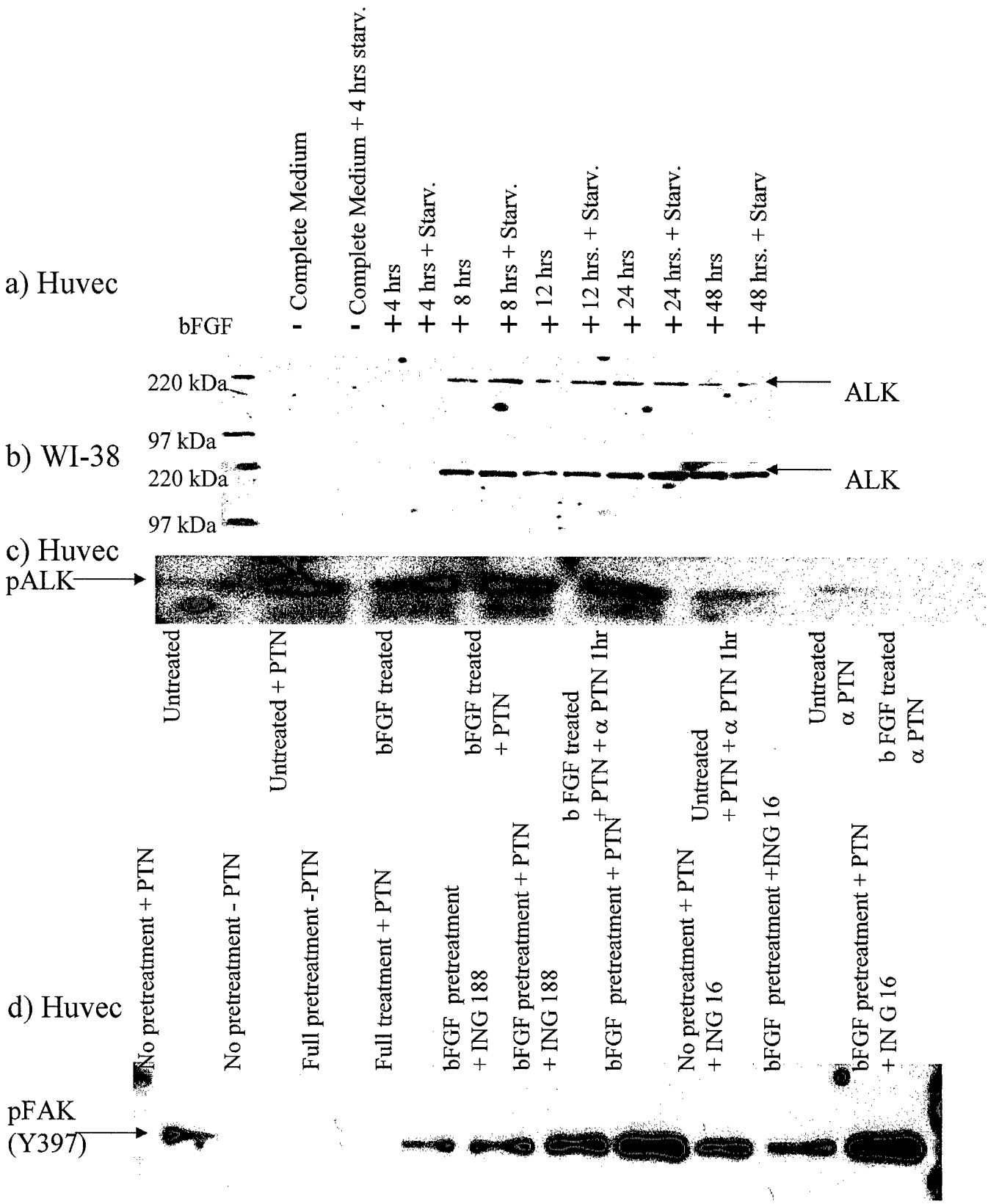


Figure 3 Functional ALK is upregulated in human endothelial cells and normal fibroblasts upon bFGF treatment. a) and b) the cells were starved for 16 hrs than treated according to the legend with bFGF for different periods. The cells were starved for a further treatment. c) Huvecs were treated for 8hrs, starved for two hours and then challenged with PTN± a blocking PTN antibody. d) Huvecs were treated as previously but a 1 hr pretreatment with the inhibitors

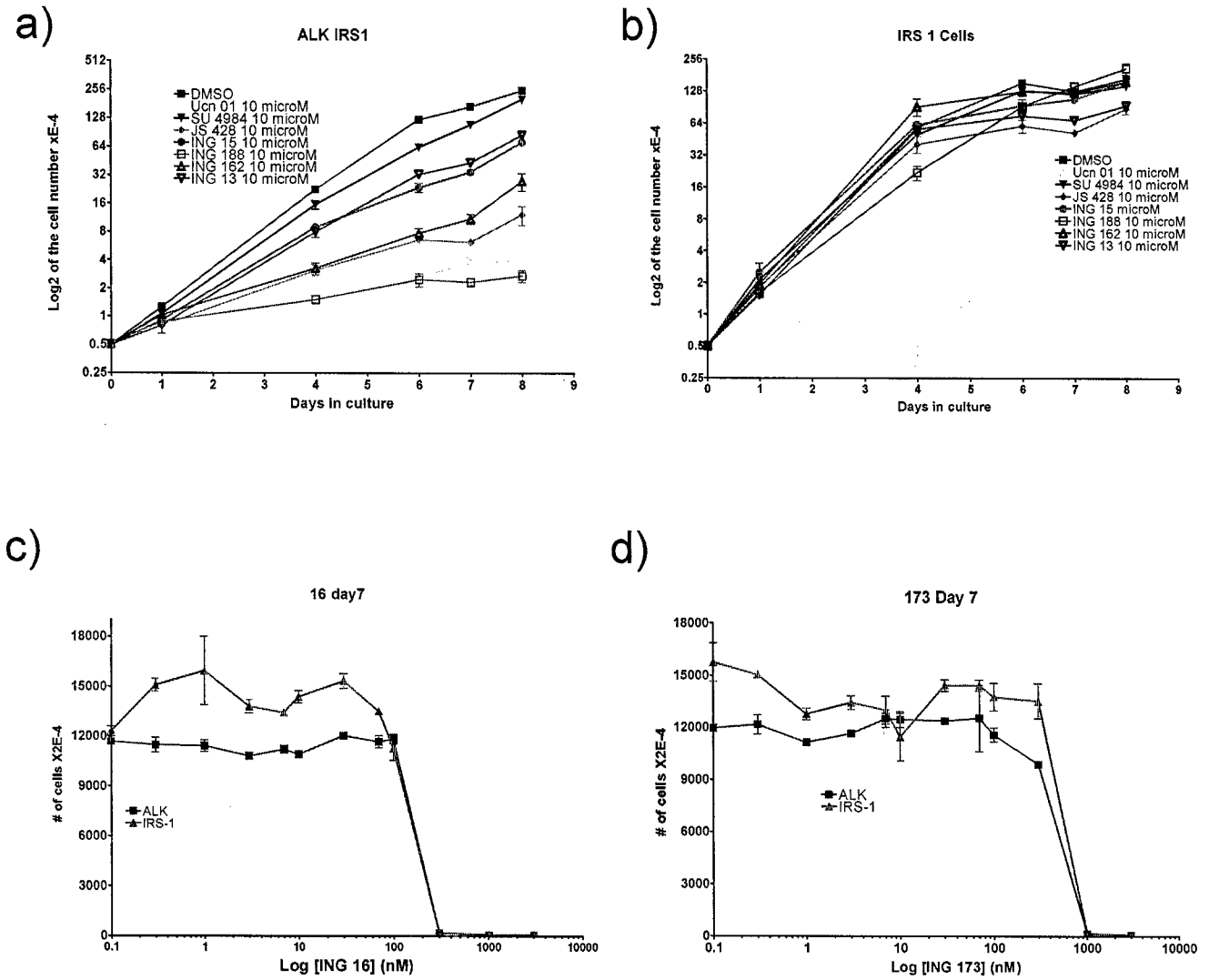


Figure 4. The effect of ALK inhibitors on ALK transfected 32 D cell growth.
a) and b) growth curves for 32 D cells transfected with ALK and IRS1 (which confers cytokine independent growth properties); the control cells (IRS-1 transfected) are still cytokine dependent in their growth so that the ALK was the cause of cytokine independent growth. The inhibitors were solved in DMSO which vas the vehicle treatment. UCN 01 is a staurosporine derivative and was our positive control and SU 4964, a PDGF inhibitor as a specificity control.
c) and d) Dose response curves showing the efficacy of two other inhibitors, ING 16 and ING 173, which both have an IC_{50} lower than 1 micromolar.

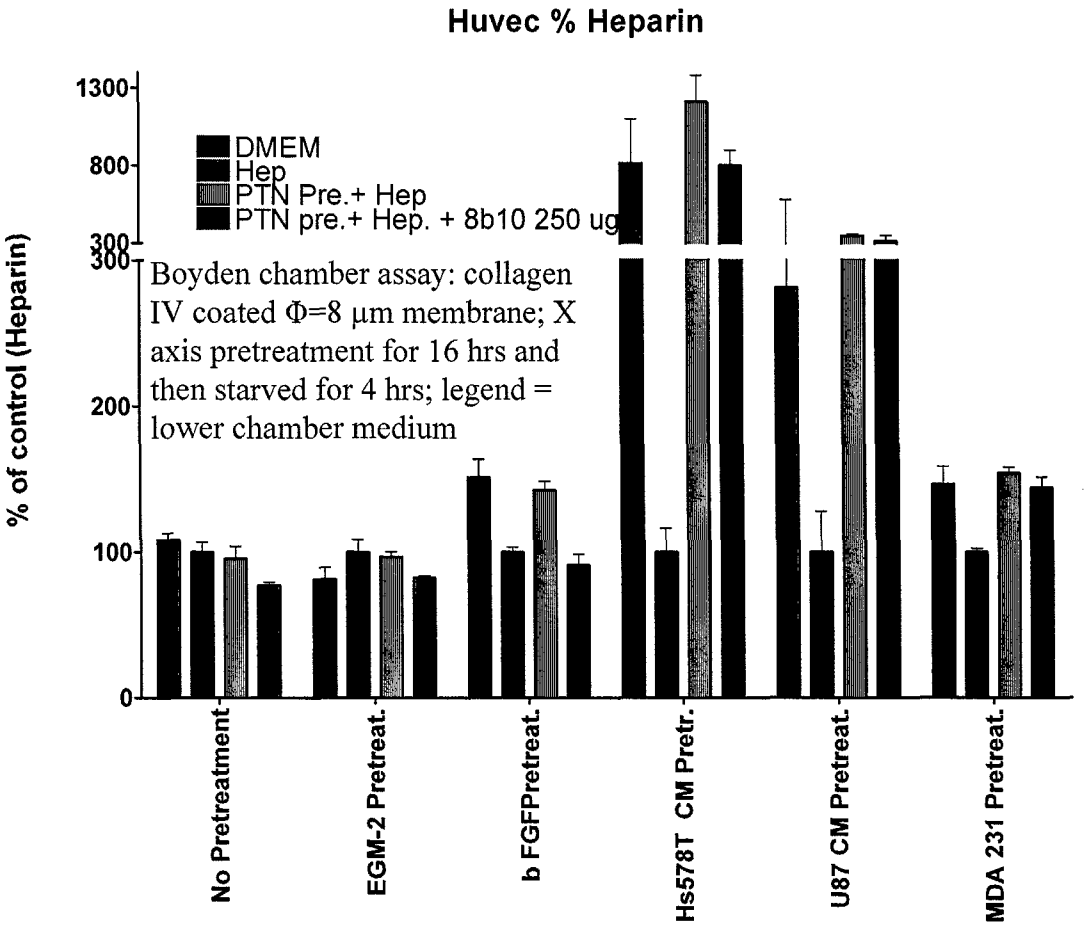


Figure 5.The migration of Huvecs can be blocked by the ALK blocking antibody 8b10

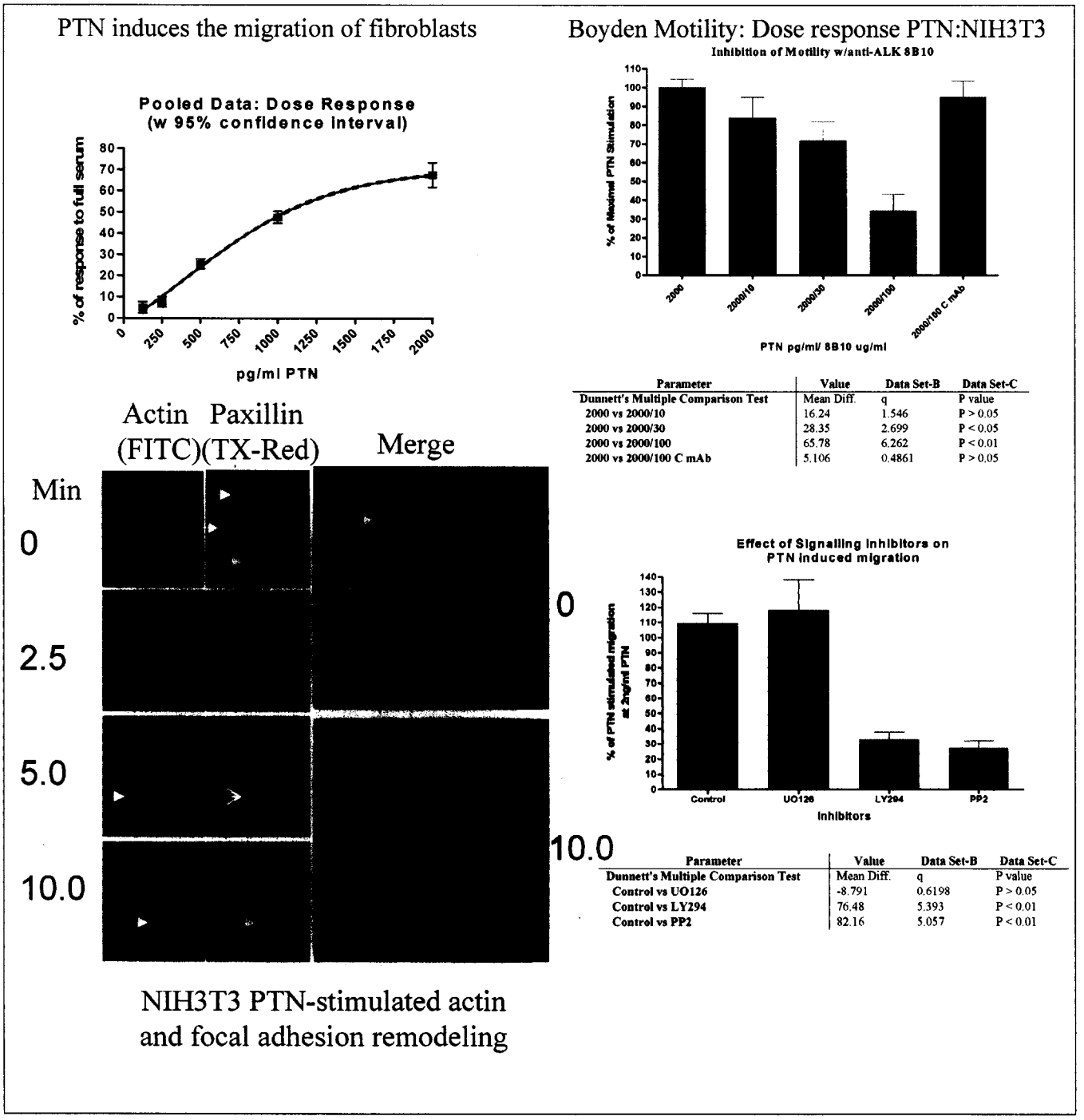
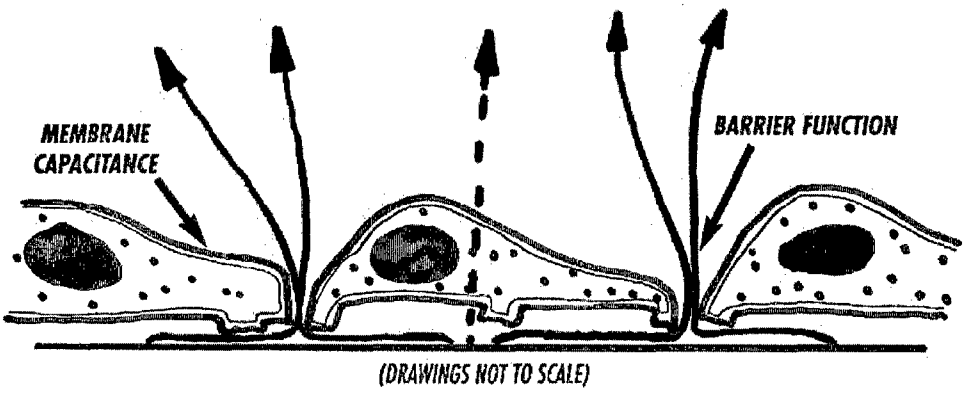
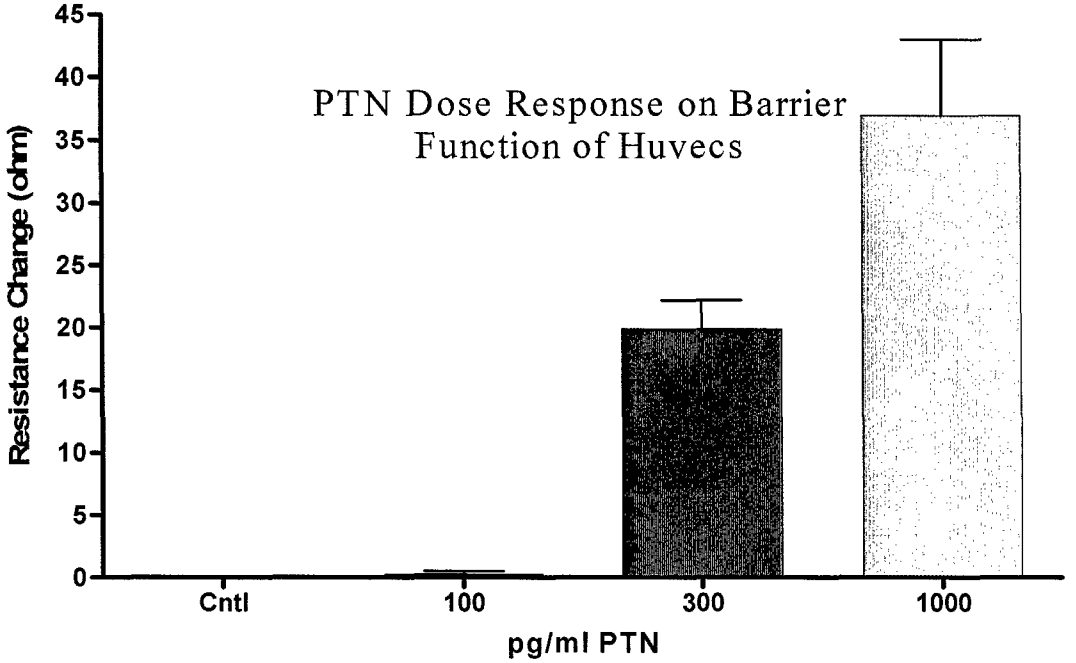


Figure 6. The effect of PTN/ALK axis on the fibroblast cell line NIH 3T3



ECIS principle



Parameter	Value	Data Set-B	Data Set-C
Tukey's Multiple Comparison Test	Mean Diff.	q	P value
Cntl vs 100	-0.2500	0.07719	P > 0.05
Cntl vs 300	-19.90	6.145	P < 0.05
Cntl vs 1000	-36.95	11.41	P < 0.01
100 vs 300	-19.65	6.067	P < 0.05
100 vs 1000	-36.70	11.33	P < 0.01
300 vs 1000	-17.05	5.265	P > 0.05

Figure7. PTN reduces the barrier function in Huvecs

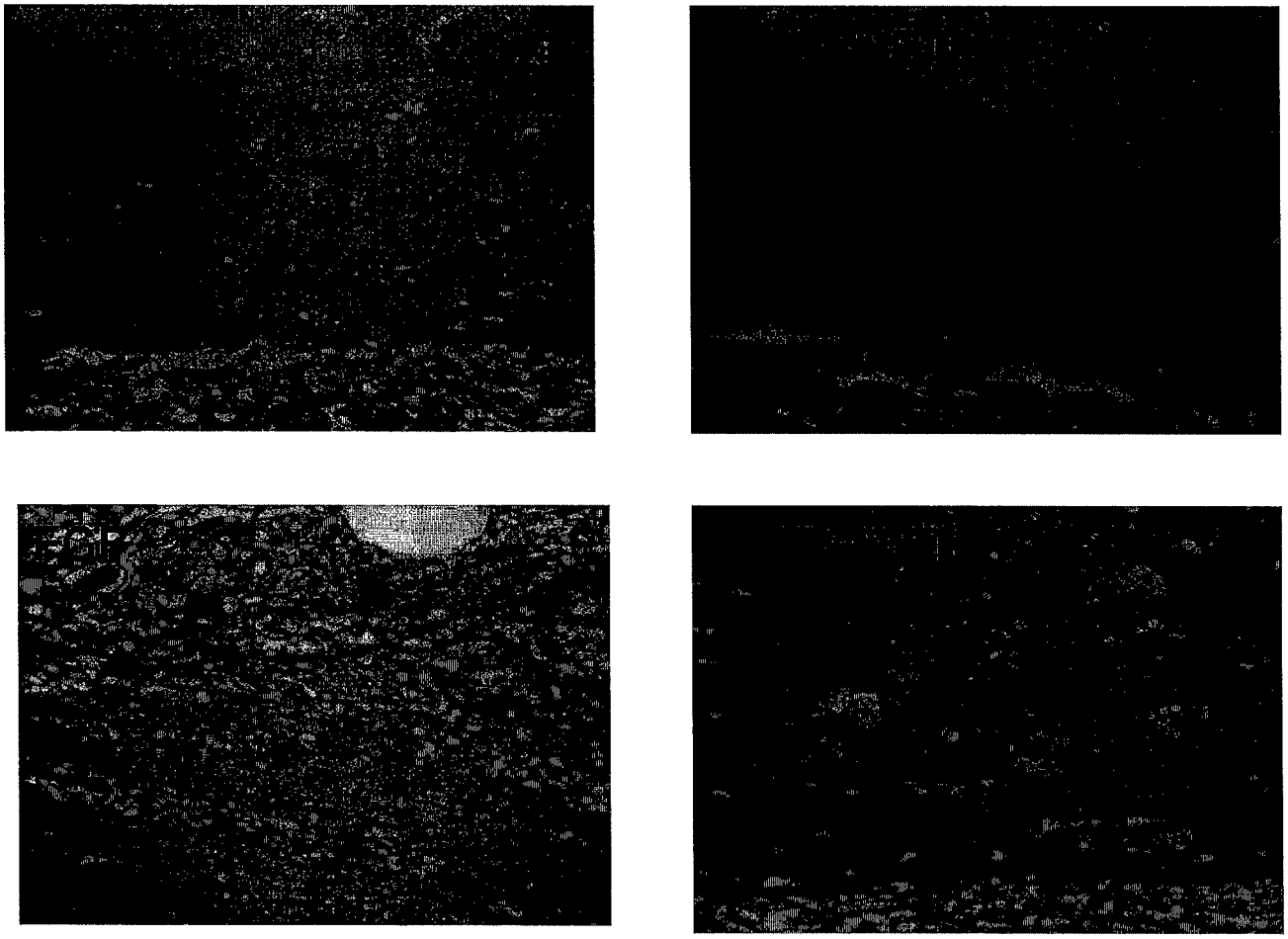
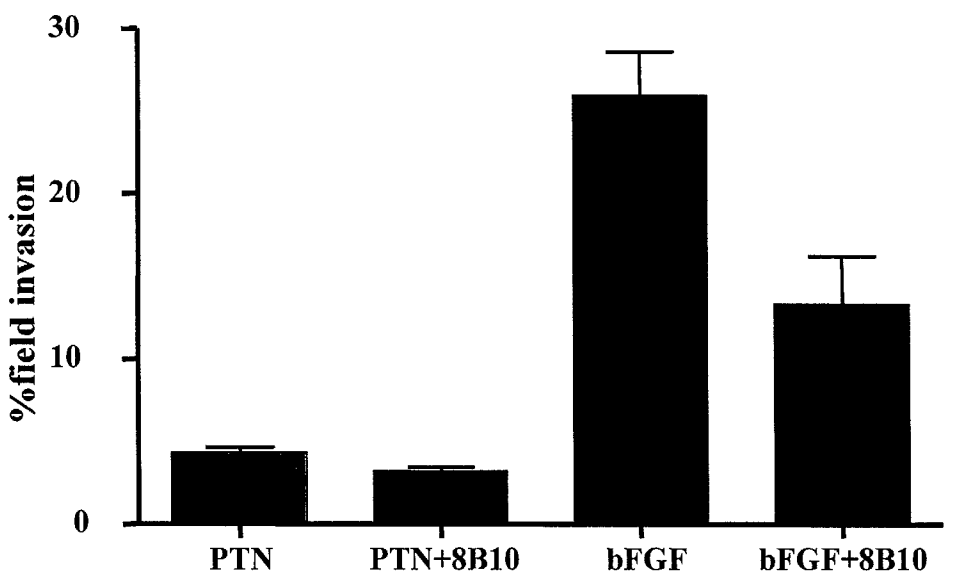


Figure 8. The blocking of neoangiogenesis caused by either PTN or bFGF in matrigel plugs



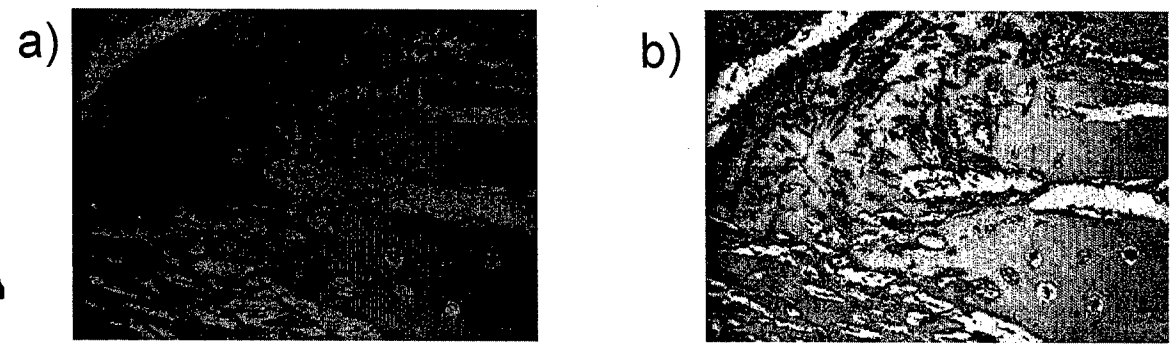


Figure 9. ALK is over expressed in the same region of active proliferation of endothelial cells. a) PCNA IHC staining of matrigel plug. b) In situ hybridization for ALK