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<b>13. ABSTRACT (Maximum 200 Words)</b> Pleiotrophin is growth factor that has mitogenic and anti-apoptotic effects in a variety of cell lines. It is also a positive regulator of tumor angiogenesis. PTN is expressed in breast cancer cell line and in primary tumor specimens as well as in fibroblasts and endothelial cell. In order to understand the biological and biochemical effects of PTN, we uncovered the PTN receptor anaplastic lymphoma kinase (ALK). Our goal was to study the signal transduction pathways for ALK/PTN axis and the modulation of ALK in stromal cells. ALK is activated by PTN and, sequentially, activates an array of second messengers in various cell lines. We identified some of the interaction partners of ALK that participate in signal transduction. In stromal cells, ALK could be upregulated by growth factors and conditioned media from an array of cancer cells. Upregulated levels of ALK in HUVEC render these cells more susceptible to PTN and promote angiogenesis. Moreover, we tested the functional consequences of downregulation of ALK by si RNA. This approach, coupled with designing small molecule inhibitors for ALK would contribute to blocking of the activation of ALK in stromal cells and promotion of tumor growth in response to PTN secreted from human mammary cancer cells.
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## Introduction

Malignant development of tumors is at least in part due to deregulation of polypeptide growth factor gene expression. The growth factors produced act on both epithelial and mesenchymal cells in the normal development of the mammary gland to regulate such processes as branching and ductal elongation, lobuloalveolar development and lactation. The delicate balance of paracrine stimulation is altered and/or autocrine stimulation may occur (1). However, the pathways by which these growth factors lead to breast cancer, as well as all the players involved, is poorly understood. One of the mechanisms proposed for tumoral development is angiogenesis. Indeed a solid tumor cannot grow more than millimeters without nourishing blood supply; the newly formed blood vessels also provide a route for metastasis (2). In breast cancer there is a direct correlation between blood vessel density in primary tumors and their metastasis.

### **The role of pleiotrophin in tumor development**

A number of heparin-binding growth factors, comprising vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), have been implicated in tumor growth, angiogenesis, and embryonic development. One such growth factor, secreted by the human cancer cell line MDA-MD 231 (3), is pleiotrophin (PTN). This growth factor has a restricted time and tissue dependent expression pattern in normal development (4-7). PTN promotes neurite outgrowth activity, has mitogenic activity on fibroblasts and epithelial cells as well as on endothelial cells and therefore it may contribute to the metastatic phenotype as a tumor growth factor and as an angiogenetic factor. Moreover, targeting PTN, either by ribozymes (8) or by transfection of mutant *Ptn* cDNA that leads to the formation of inactive PTN dimers (9), inhibited human breast cancer growth.

### **A newly discovered protein as a receptor for PTN**

The biological activity of PTN affects a large palette of cell types but the mechanism of action could not be adequately studied because its receptor was not known. Our laboratory identified the receptor for pleiotrophin as a transmembrane receptor tyrosine kinase as a novel member in the insulin receptor family, anaplastic lymphoma kinase (ALK). In SW13 human adrenal carcinoma cell line the receptor is phosphorylated in response to PTN in a time dependent manner. Along with the tyrosine (Y) phosphorylation of the receptor, there are other proteins Y phosphorylated, some of which were identified by their apparent molecular weight and previous results as SHC and ERK1 & 2.

Interestingly, the mRNA for ALK is expressed in eight of the eighteen breast cancer cell lines. Intriguingly, these cell lines have lost their sensitivity to estradiol (ER-) and are established from invasive types of tumors. ALK was also expressed in 9 of 13 breast cancer samples, as well as other malignant tumors (10). Moreover, the proliferation rate of Hs578T cells (which express both PTN and ALK) was reduced by 60% by stable of expression ALK targeted ribozymes that reduced the ALK mRNA level by the same percentage.

In the last years of the proposal we decided to investigate the biochemical and biological effects of the PTN -ALK interaction. Where pursuing these studies, we observed an important phenomenon, namely upregulation of ALK in stromal cells, thus evading the normal process of apoptosis and providing support for the tumor growth.

The study of this fact coupled with discovering the signal transduction pathways constitute the object of this report.

The long-term goal of our work is to decipher the signal transduction pathways of the ALK, how does this modulate the malignant phenotype of the cells that harbor ALK and how can this process be blocked.

### **Proposal Body**

#### **Proposal Body**

In the approved, redirected, Statement of Work three tasks were outlined.

- Task 1.** Expression status of PTN and HERV-PTN fusion transcripts in human mammary carcinoma (month 8 to 12).
- Task 2.** Characterization of the mechanism(s) of HERV-PTN fusion transcript expression in human breast cancer cell lines (month 1 to 30).
- A. Transcriptional activity of the HERV-derived PTN promoter in breast cancer cell lines (month 1 to 18).
  - B. Identification of cis-elements in the HERV-derived PTN promoter (month 14 to 30).
  - C. Examine whether posttranscriptional regulatory mechanisms are involved in the HERV-PTN fusion transcript expression in human breast cancer cell lines (month 28 to 36).
- Task 3.** Identification and characterization of the PTN receptor (month 28 to 46).
- A. Identification of the receptor for PTN as anaplastic lymphoma kinase (months 28-36)
  - B. Study the signal transduction for the PTN- ALK axis (months 36-46)
  - C. Study the interaction between stromal cells expressing ALK and cancer cells. (months 40-46).

The data obtained will offer a more comprehensive picture of the complex interaction between tumor cells, and stroma and also uncover potential targets for therapy.

**Task 1:** The goal of our studies regarding Task 1 was to study the expression of PTN and HERV-PTN fusion transcript in human mammary carcinoma.

Progress regarding Task 1 was documented in the first Annual Report (1 Sep 1999 – 31 Aug 2000).

**Task 2:** The focus of Task 2 was the analysis of the regulatory elements of the HERV-derived PTN promoter. Specifically we are interested in regulatory

cis-elements of this promoter and their interaction with the transcription factors that bind to these elements.

Completion of task 2 was documented in the second annual report (1 Sept. 2000- 31 Aug. 2001).

**Task 3** We intended to identify and characterize of the PTN receptor. We detected ALK as a receptor for PTN , identified major signaling proteins that are activated as a response to ALK – PTN interaction.

Progress towards task 3 was reported in the third annual report (1 Sept. 2001-31 Aug. 2002).

In this report we will document the progress towards

- Task 3.** Identification and characterization of the PTN receptor (month 28 to 46).
- B. Study the signal transduction for the PTN- ALK axis (months 36-46)
  - C. Study the interaction between stromal cells expressing ALK and cancer cells. (months 40-46).

#### **Expression of ALK in various cell lines**

As presented in the previous report, we identified ALK in the normal breast fibroblasts and in endothelial cells. Therefore we intended to probe ALK expression in various breast cancer cell lines as well as in other cell lines. The cell lines tested were: WI-38 (human normal lung fibroblasts), MDA-MB-231 (human breast cancer cells), Hs 578T (human breast cancer), PC3, DU 145 and LnCaP (human prostate cancer cells). As presented in figure 1, the level of expression varied between the cells tested. ALK was not detected in other breast cancer cell lines tested (e.g. MCF-7) (data not shown). It is worth mentioning that in the breast cancer lines presented (MDA-MB-231 and Hs 578T), the ligand for ALK, PTN, is expressed and secreted. This fact indicates an autocrine action of PTN for these cells.

#### **Growth factors and conditioned media from various cell lines modulate ALK expression in endothelial cells**

An intriguing observation regarding ALK expression in endothelial cells (HUVEC) was that, after initial starvation in the basal EBM-2 medium (Clonetics, Walkersville, MD) supplemented with 0.5% FBS and treatment with growth factors or conditioned media from various cell lines, ALK is induced. As shown in figure 2, ALK expression is induced 2-4 fold over the level considered basal (treatment with EBM-2 + 0.5% FBS). The treatments used were: bFGF (10ng/ml), IGF (40 ng /ml) EGF (100ng/ml), PDGF (40 ng/ml). EBM-2 and EGM-2 were prepared according to the manufacturer's indication. We used conditioned media (at a 1:1 ratio with EBM-2) from the following cell lines: SW-13 (human adrenal carcinoma), SW-13/PTN (same as W2-8)

(SW-13 cells stably transfected with PTN cDNA), MDA MB 231, Hs 578T, and MCF-7 (human breast cancer cell lines), PC-3 and DU-145 (human prostate cancer cell lines) and U 87 MG (human glioblastoma). It is remarkable that a variety of growth factors are inducing the ALK expression, either by themselves or in combination (as in conditioned media treatments). However, PTN was not able to induce its own receptor as demonstrated by the use of W2-8 cells, which express a high amount of PTN in their conditioned media (~8 ng/ml).

**Phosphorylation of ALK in response to PTN treatment is enhanced in endothelial cells pretreated with selected growth factors.**

In order to bolster our findings that ALK is induced by pretreatment with bFGF and IGF I we pretreated the HUVEC with bFGF, IGF I, or with complete EGM-2 medium (figure 3) and then treated with PTN, MK, control medium from SW 13 cells, as well as Epidermal Growth Factor (EGF) and Vascular Endothelial Growth Factor (VEGF) as negative controls. PTN but not other ligands triggered an ALK phosphorylation (as detected by phospho ALK antibody). The phosphorylation was most pronounced in HUVEC pretreated with bFGF. When HUVECs were pretreated with EGM-2 (complete medium containing bFGF and IGF-I and other factors), EBM-2 + 0.5% (Basal Medium), or IGF-I, ALK phosphorylation was noticeable but at a lesser extent.

**Down regulation of ALK has functional consequences.**

The reverse of the induction of ALK in cells that express this receptor is down regulation. To investigate the biological consequences of the modulation of ALK we transiently transfected siRNA targeting *Alk* mRNA. The use of siRNA is increasingly widespread in experiments that effectively block the expression of proteins (REFERENCE). We transfected siRNA targeting ALK in SW13 cells that are stably transfected with *Ptn* cDNA (W2-8 cells). Thus, these cells express both the ligand (PTN) and its receptor (ALK expressed endogenously) so that an autocrine loop is formed. These cells would grow in soft agar spontaneously as opposed to the parental cells (SW13) which express just ALK and need PTN added exogenously in order to grow in an anchorage independent manner. In figure 4 a), we tested the ALK down regulation by Western immunoblot and compared it with actin levels. As a control we used actin, which is not influenced by ALK siRNA or by negative control siRNA. The levels of ALK were down regulated significantly starting with as low a concentration as 40nM. The cells containing the ALK siRNA or the control siRNA were seeded in soft agar and the number of colonies formed was counted. The cells transfected with ALK siRNA formed significantly fewer colonies than the cells transfected with the control siRNA. The ALK siRNA did not have a toxic effect on W2-8 cells because, when triggered with basic FGF, another growth factor that does not signal through ALK, the cells were able to grow in soft agar. The experiment underscores the significant role that PTN/ALK axis is playing, at least in SW 13 cells.

### **ALK can phosphorylate other membrane receptors**

The closest ALK family member is the leukocyte tyrosine kinase (LTK). This receptor has a relatively short extra cellular domain (11) that makes it unlikely that a ligand will be found for it. However, the receptor has an active kinase domain that it has to be activated by external or internal factors. One of the possibilities is that ALK might activate LTK by phosphorylating it due to an initial triggering by PTN binding to ALK. Therefore, we tested the hypothesis that ALK (immunoprecipitated from SH-SY-5Y neuroblastoma cell line treated with PTN or untreated) would phosphorylate LTK (immunoprecipitated from Cos 7 cells transiently transfected with V5 epitope tagged LTK) (figure 5). As can be noted from the figure, ALK is able to phosphorylate, *in vitro*, LTK immunoprecipitated from Cos 7 cells. A slight increase in LTK phosphorylation is observed in lane 2; also autophosphorylation of ALK can be noted. As controls lanes 3 and 4 show a low level of LTK phosphorylation which could be generated from endogenous LTK that was co-immunoprecipitated with ALK. The phosphorylating abilities of LTK are highlighted in lane 5, which contains LTK immunoprecipitated from Cos 7 cell transfected with V5 tagged LTK. The facts presented suggested that LTK might act as a co receptor for ALK and influence the ALK signaling.

### **ALK interacts with an array of proteins which potentially modulate its signaling**

Recently we employed a novel technique to detect proteins that interact with ALK and may modulate its signaling properties: Liquid chromatography/Mass spectrometry. Due to the conditions implicated in the analysis (12) which require a considerable amount of protein, we were prompted to immunoprecipitate ALK from HEK 293 cells transfected with His/myc epitope tagged ALK. Overexpression of ALK triggers cross-phosphorylation of the receptor so as any proteins associated with ALK would mimic the situation of the active ALK. As a control we used an ALK mutant that has an inactivated kinase domain. As may be noted from figure 6, there are differences between the proteins that are immunoprecipitated from ALK and the kinase dead mutant. The protein bands were excised and digested with trypsin. The tryptic peptides are then loaded on a liquid chromatograph and the effluent was fed to a mass spectrometer with peptide sequencing capabilities. Preliminary results detected a previously unknown phosphatase to associate with active ALK. The interaction was further detected biochemically (data not shown).

### **Key research accomplishments**

- We detected ALK expression in fibroblasts, breast cancer cells as well prostate cancer cells.
- ALK expression in endothelial cells is enhanced by pretreatment with basic FGF or conditioned media from breast cancer cells, prostate cancer cells and neuroblastoma.
- We uncovered the functional consequences of the down regulation of ALK in a system dependent of the PTN/ALK system.
- We demonstrated that ALK could phosphorylate LTK, a related membrane receptor.
- We identified interaction partners for ALK that may modulate the signaling.

### Reportable Outcome:

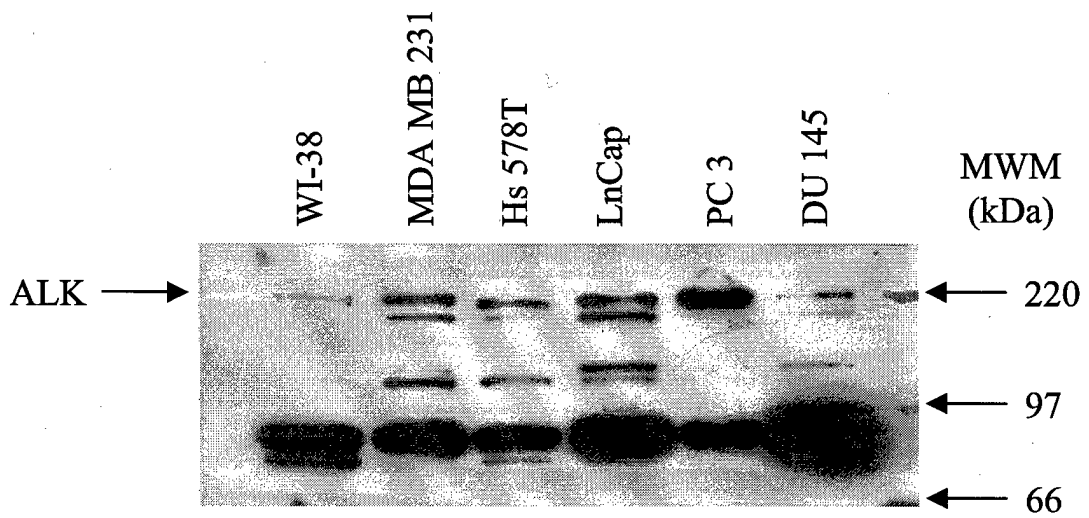
- Detection of growth factors that modulate ALK
- Identification of heteromeric interaction between ALK and other membrane receptors
- Discovery of previously unknown ALK interaction partners by novel techniques
- Gerald E. Stoica, Angera Kuo, John T. Lahuisen, Anton Wellstein, "Expression and modulation of ALK in stroma of PTN expressing tumors", manuscript in preparation.

### Conclusions:

In the last period of the proposal we continued the study of the PTN receptor and the signal transduction mechanisms triggered by the PTN and ALK interaction. We detected ALK expression in breast cancer cells, in stromal cells (endothelial and fibroblasts) as well as in prostate cancer cells. Moreover, in human umbilical vein endothelial cells, ALK is induced by basic FGF as well as by conditioned media from breast cancer cell lines, prostate cancer cell lines or a human glioblastoma. This finding is very important because it substantiates the hypothesis that cancer cells enroll the stroma for tumor progression and possibly metastasis. Also, up regulation of ALK enhances the PTN antiapoptotic and proliferative signals. We started to investigate which are the proteins that are associated with ALK in the activated as well as basal state, in order to further investigate the signal transduction pathways. The completion of the study would enable us to have a solid basis for an intelligent drug design.

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**Figure 1. ALK is present in stromal cells and cancer cell lines**

ALK is detected in lysates of WI-38 (human fibroblasts), MDA MB 231, Hs 578T (breast cancer cell lines) as well as prostate cancer cell lines (LnCaP, PC 3, DU 145).

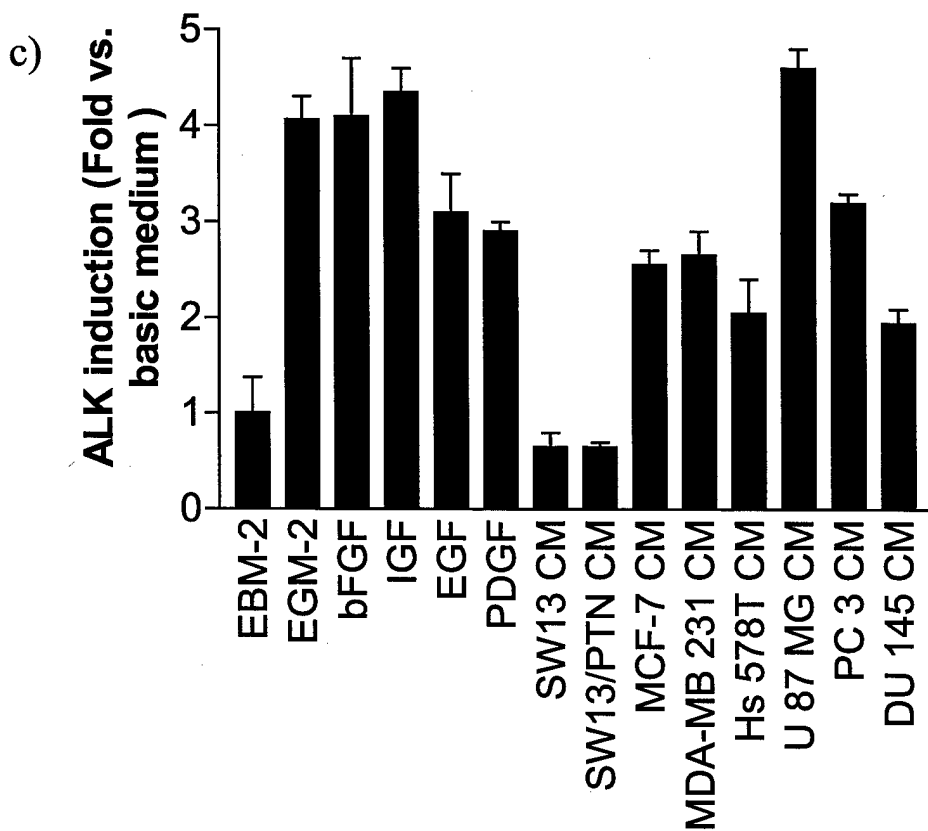
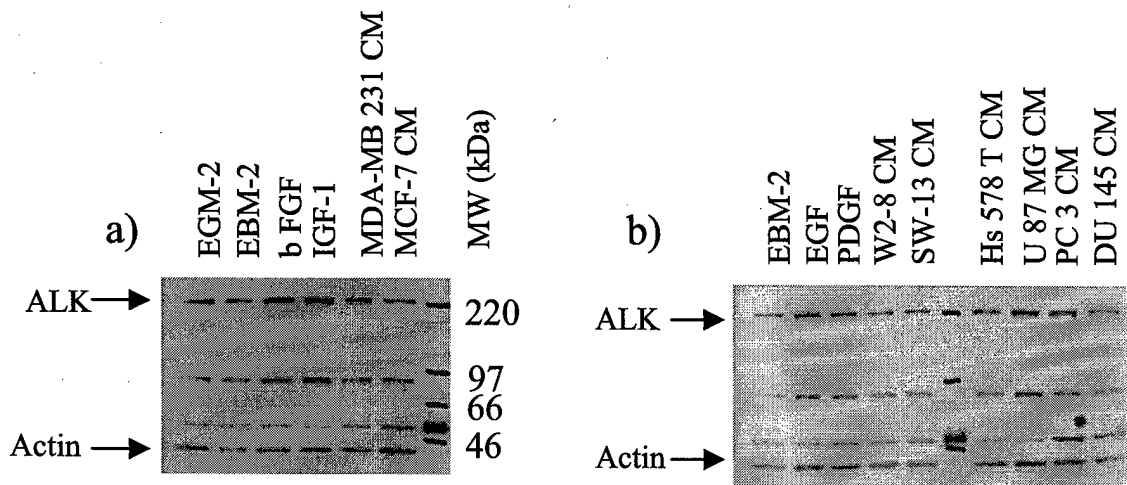
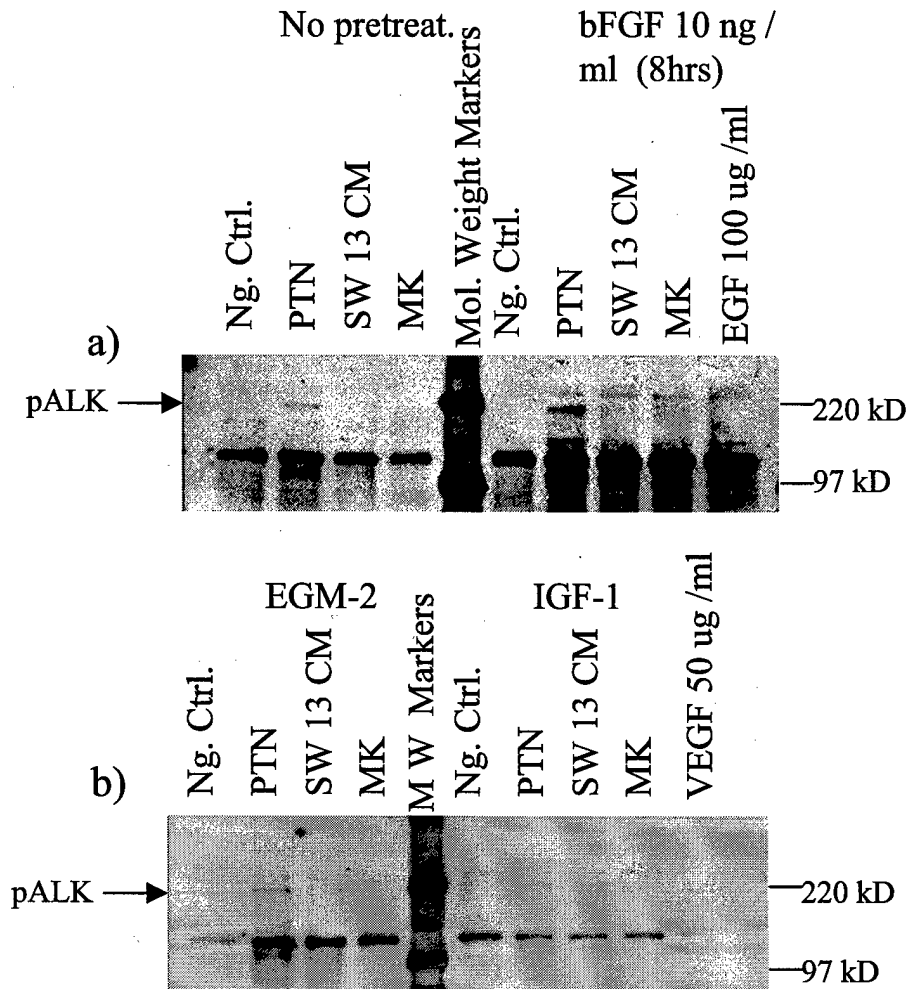


Figure 2. Induction of ALK in HUVECs

a), b) Huvec cells were treated for 8 hrs with the indicated growth factors or with conditioned media from two breast cancer cell lines and lysates were used for the SDS PAGE and Western blotting detection of ALK c) Quantitation of the ALK induction in HUVECs by various treatments.



**Figure 3. Activation of ALK in Huvec cells as a result of PTN binding**

a) Tyrosine phosphorylation of ALK is enhanced upon pretreatment with bFGF (10 ng/ml) for 8 hours when the cells are stimulated for 10 minutes with PTN, but not with 0.9 M NaCl (neg. ctrl.), SW 13 CM or midkine (MK). b) Pretreatment with the complete growing medium (EGM-2), which contains b FGF and IGF-1, and with IGF 1 40 ng/ml also show activation of ALK by PTN albeit at a much lower rate.

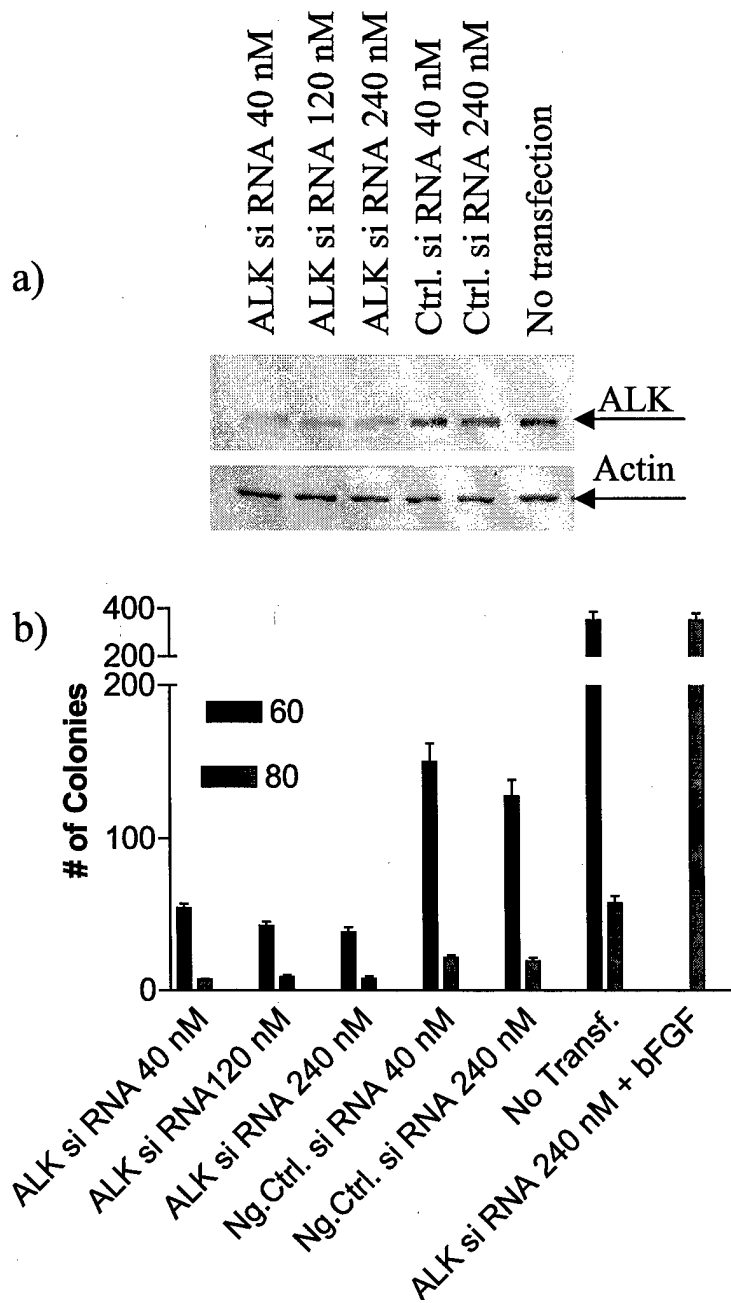


Figure 4. Functional consequences of ALK downregulation by siRNA

a) SW 13 cells expressing both endogenous ALK and stably transfected PTN were transiently transfected with ALK siRNA to lower the ALK expression. The western blot is representative for ALK down regulation after 36 hrs from the transient transfection b) Anchorage independent growth of the same cells as above was quantified. Black bars- colonies larger than 60  $\mu\text{m}$ ; gray bars- colonies bigger than 80  $\mu\text{m}$ .

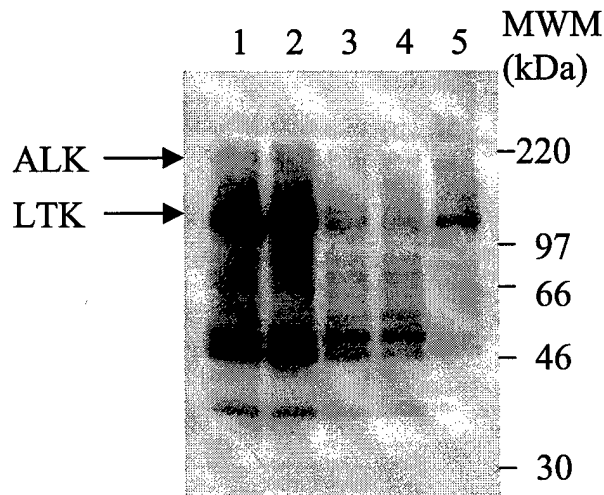


Figure 5. ALK can phosphorylate LTK *in vitro*

In the *in vitro* phosphorylation assay, ALK was immunoprecipitated from SH-SY-5Y cells which were treated with control (1) or PTN (2) containing medium and let react with LTK immunoprecipitated from Cos 7 transfected with LTK V5 tagged. Lanes 3 (control) and 4 (PTN treated) represent the ALK immunoprecipitated from SH-SY 5Y cells. Lane 5- LTK immunoprecipitated from Cos 7 LTK transfected cells.

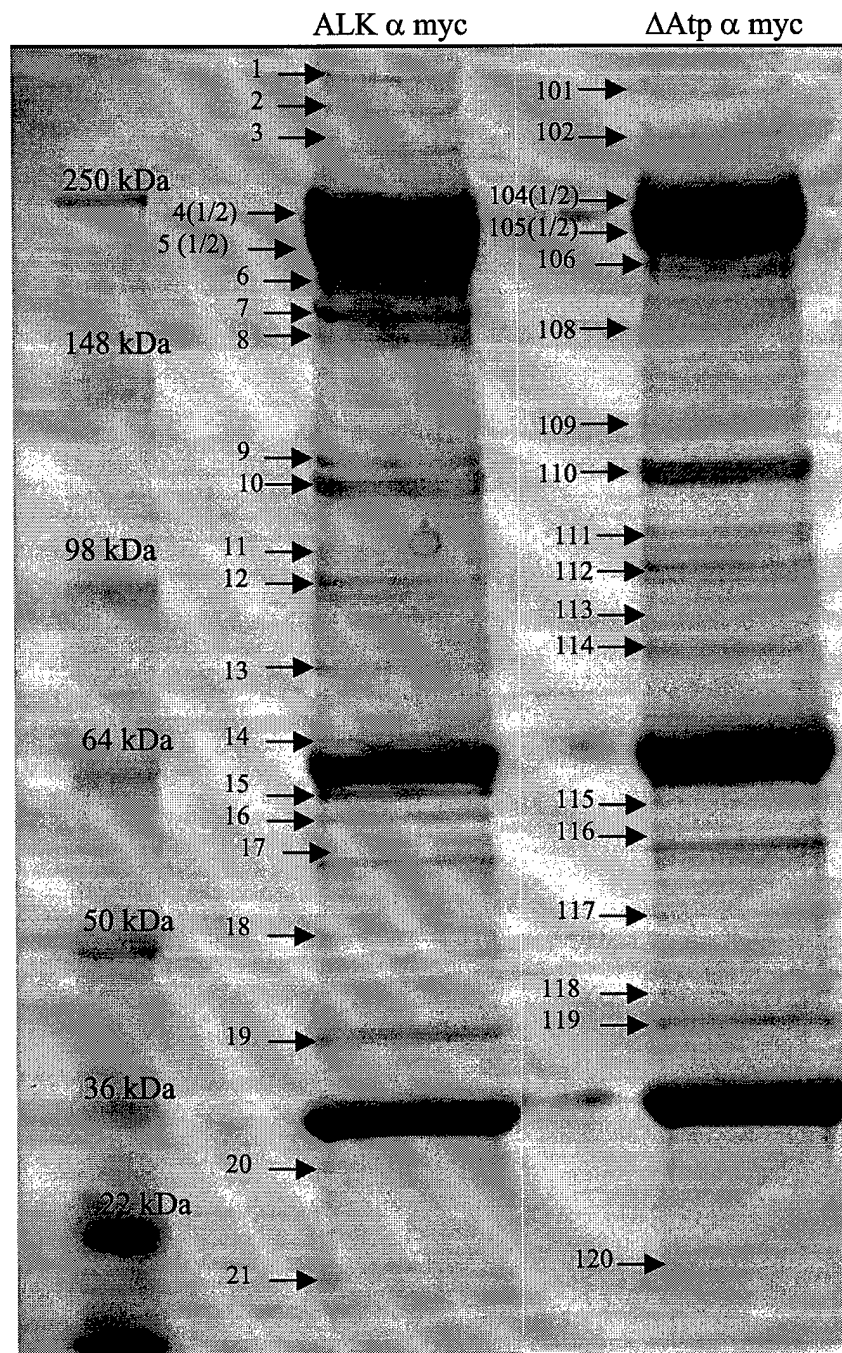


Figure 6. Differential coimmunoprecipitation of proteins related to the phosphorylation status of ALK.

ALK or the  $\Delta$  ATP mutant of ALK were overexpressed in HEK 293 cells and then immunoprecipitated. The complex mixture of proteins was separated by SDS-PAGE and selected proteins were analysed by LC/MS-MS.