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In the past year two major findings with respect to the role and regulation of the growth factor pleiotrophin (PTN) were contributed by my laboratory: (1) We discovered that in the human PTN gene a tissue-specific promoter was generated by the germ line insertion of an endogenous retrovirus some 15 to 25 million years ago. This is the first report of a retroviral insertion contributing a tissue-specific promoter in a human gene and only the second human gene that was reported to be altered in its expression pattern by retroviral elements. (2) We demonstrated that the expression of PTN can be rate-limiting for tumor growth, invasion, angiogenesis and metastasis. This was demonstrated by PTN depletion through ribozyme expression in defined human tumor models. Both findings were published in two back-to-back articles in PNAS (93:14753-64; 1996). In addition to these two major findings, we observed that the newly discovered promoter responds to steroid hormones. Furthermore, we generated data that allow to study the immediate signal transducting activity of PTN and thus allow to assess to what extent mutated PTN is able to interfere with this signalling.

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Jan 10, 97

PI - Signature Date

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INTRODUCTION

Under our proposal we study the role of the growth factor pleiotrophin (PTN). We hypothesize that PTN is an essential, rate-limiting growth factor for PTN-positive breast cancers. Our experiments are designed to address this hypothesis. We were the first laboratory to purify PTN from human cancer cells (MDA-MB 231 breast cancer cell line) [1]. We were also the first laboratory to generate biologically active recombinant PTN [2] and to demonstrate its potential role as a tumor growth and angiogenesis factor [2]. Furthermore, we showed its distinct expression in human breast cancer samples [2] and published the genomic structure of the human PTN gene first [3].

We propose that the secreted polypeptide growth factor pleiotrophin (PTN) plays a major role in the growth and metastasis of breast cancer. This hypothesis is based on the high levels of PTN expression in 60% of tumor samples from breast cancer patients but not in normal tissues and on the biological effects of PTN in selected tumors models. In particular, the activity of PTN on endothelial cells indicates that PTN can serve as a tumor angiogenesis factor and its expression can thus enhance the ability of breast cancer to metastasize.In summary, our studies quoted above as well as the data published by others show:

(1) PTN is a secreted growth factor expressed in a number of human breast cancer cell lines.
(2) PTN stimulates endothelial cells and can act as a tumor angiogenesis factor [4].
(3) PTN can support tumor growth of non-tumorigenic SW-13 cells or fibroblasts [5].
(4) PTN mRNA is upregulated by retinoic acid treatment.
(5) PTN mRNA is found at high levels in 60% of samples from patients with breast cancer.
(6) PTN is not expressed in normal breast epithelium (40% of the breast cancer samples were negative for PTN inspite of the fact that they obviously contain breast epithelial tissue).

OVERVIEW OF THE GOALS:
In our studies, we elucidate the role of PTN and the hormonal regulation of its activity, with the ultimate goal to develop novel therapeutic strategies.
In particular,
(1) we study hormonal regulation of PTN
(2) investigate whether PTN expression can support tumor growth
(3) generate mutated PTN protein for functional studies and
(4) target PTN mRNA to repress production of PTN and thus inhibit tumor growth

OVERVIEW OF MAJOR FINDINGS IN THIS REPORT:
In this report cycle, I am pleased to present data from a recently completed series of experiments which were prompted by the surprise discovery little more than a year ago that an endogenous human retrovirus had inserted itself into the human PTN gene and directly controls tissue-specific expression of this gene. This data was submitted in the summer of 1996 to PNAS through the "public pathway" and was accepted for publication in the fall (Dr. Duisberg from UC Berkley communicated the paper in the December issue of PNAS [6]).
Due to this exciting finding of a functional retroviral regulator in a human gene, we focussed our efforts in this area and were also able to demonstrate that regulation of the human PTN gene by steroid hormones resides also within this retrovirus-derived new promoter region. Although these studies are still preliminary, they hold great promise to unravel mechanistically the hormonal regulation of human PTN.

In parallel functional studies, we demonstrated that the retroviral-driven transcript codes for the wild-type PTN protein. We then demonstrated the role of the PTN protein for tumor invasion and angiogenesis by depletion of the PTN mRNA in tumor cells using ribozymes. In additional studies completed in this report cycle, we also demonstrated that PTN contributes not only to tumor invasion and angiogenesis but also to metastasis. We were able to show that in a "gene dose-dependent" manner even small reductions (<25% reduction) of PTN mRNA in PTN-positive tumor cells already affected their angiogenic, tumorigenic and metastatic phenotype. This set of data was also submitted to PNAS through the "public pathway" and published back-to-back with the retrovirus-PTN paper (Dr. Folkman from Harvard University communicated the paper [7].)

OVERVIEW OF METHODS:

For goals (1) and (2) in the present year we used gene cloning and characterization of a newly discovered retroviral promoter in the human PTN gene to lay the basis for mechanistic studies of hormonal regulation. The methods are described in detail at the end of the respective section.

For goal (3) we set up signal transduction assays to assess the activity of wild-type relative to mutated PTN.

For goal (4) different constructs blocking PTN production were used.

BODY

Specific Aim (1): Regulation of pleiotrophin (PTN) by hormones

Background:
Hormones and growth factors define the capacity of human breast cancer to grow and metastasize. One of the essential requirements for the development of breast cancer are circulating steroid hormones and one of the most widely used drug therapies of breast cancer with the anti-estrogen tamoxifen is based on this fact. Furthermore, growth factor gene expression can supplement for hormone stimulation and thus contribute to hormone-independent cancer growth as well as to resistance to anti-hormone therapy (reviewed e.g. in [8]).

Work accomplished:
Summary: We discovered that in the human PTN gene a tissue-specific promoter was generated by the germ line insertion of an endogenous retrovirus some 15 to 25 million years ago. This is the first report of a retroviral insertion contributing a tissue-specific promoter in a human gene and only the second human gene that was reported to be altered in its expression pattern by retroviral elements. This promoter appears to confer some steroid regulation of the human gene.

1. Discovery of a retroviral insertion in the human PTN gene:
To elucidate the mechanisms that regulate expression of the human PTN gene, we examined the 5'regions of mRNAs isolated from different tissues by 5'RACE PCR (details see below under "Methods"). In particular, PTN expressed in placental tissues appeared of interest to us, since placenta is mostly derived from fetal tissues and is highly hormonally regulated. Furthermore, in situ hybridization [9] as well as Northern analysis (unpublished data) with rodent trophoblast tissues had failed to detect a signal for PTN in contrast to a strong signal in Northern blots with human placenta (see Fig. 1C). To our surprise, 5'RACE PCR clones with mRNA from placenta contained novel 5'UTR that are distinct from the previously described 5'UTR in human placental and brain cDNAs.

Sequence comparisons revealed that the novel 5'exons contained in the PTN mRNA from placenta are highly homologous to different regions of human endogenous retrovirus (HERV) type C [10-12]. Analysis of human genomic DNA revealed that the HERV fragment is inserted in sense orientation into the intron region immediately upstream of the ORF of the human PTN gene expanding this region relative to the murine gene (Fig. 1A). Low-stringency Southern blot analysis confirmed insertion of HERV also in the rhesus monkey genome and showed the lack thereof in murine genomic DNA (unpublished data). This suggests an insertion into the PTN gene between 15 and 25 million years ago, i.e. after the separation between apes and monkeys.

The most 5'HERV-derived PTN exon (UV3) is homologous to the viral 5' long-terminal repeat (LTR) region and the downstream UV2 and UV1 exons are homologous to regions of the HERV gag, pol, and env pseudogenes (70, 85, and 80% identity respectively; Fig. 1B) [10,12]. Unlike infectious C-type viruses encountered today, all of which contain a $tRNA^{PRO}$ primer binding site in their DNA [10], this prehistoric virus contains a $tRNA^{GLU}$ primer binding site as its signature (Fig. 1B).

Fig. 1. Insertion of HERV into the human PTN gene and tissue-specific distribution of HERV-PTN fusion transcripts.

A. Comparison of the genomic organization of the human and murine PTN genes. Homologous exons corresponding to the ORF (O1 to O4) or 5'UTR (U2, U1) of the murine [13] and the human gene [3,14] as well as the HERV-derived exons (UV3, UV2, and UV1) and the position of exon-specific probes are shown.

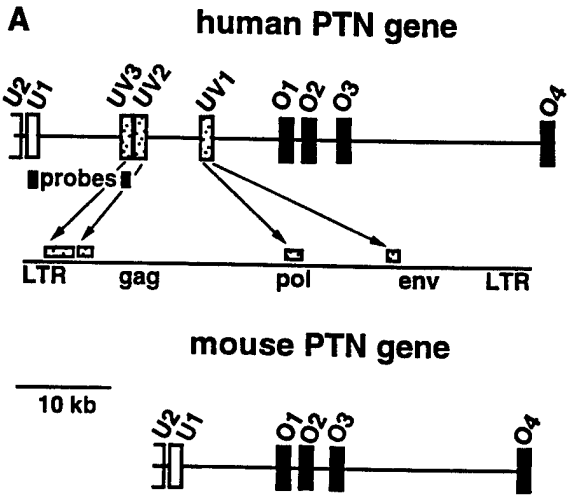


Fig 1B. -see next page- Sequence comparison between the HERV insert in the human PTN gene and type-C HERV fragments LTR8, LTR22 and HERV 4-1 [10,12] using the GCG program. Gaps (//) were introduced for optimal alignment. The PTN gene numbering system (filed by us under **GenBank accession numbers U71455 and U71456**) on the left refers to the first nucleotide of the translation start codon as +1. The viral sequence positions were taken from the respective GenBank entries for LTR8, LTR22 and HERV 4-1 (accession numbers M32219, M32220 and K02168 respectively). A 280 nt Alu sequence in the promoter region as well as CAAT and TATA boxes are underlined and intronic sequence is denoted by lower case lettering. The transcription start site (l==>) was mapped by primer extension. Similarity to retroviral elements is indicated on the right and the respective nucleotide positions separating the different elements are highlighted in the sequence. The tRNAGlu primer binding site, UV3 cDNA end (^), restriction sites used for generation of the promoter reporter constructs and the N-terminal sequence of the PTN protein are shown. The splice donor (SD) sites of UV3, UV2 and UV1 as well as the splice acceptor (SA) in exon O1 are underlined and the retroviral SD of HERV 4-1 is indicated in addition (.....).

Fig. 1B. (legend on previous page !)

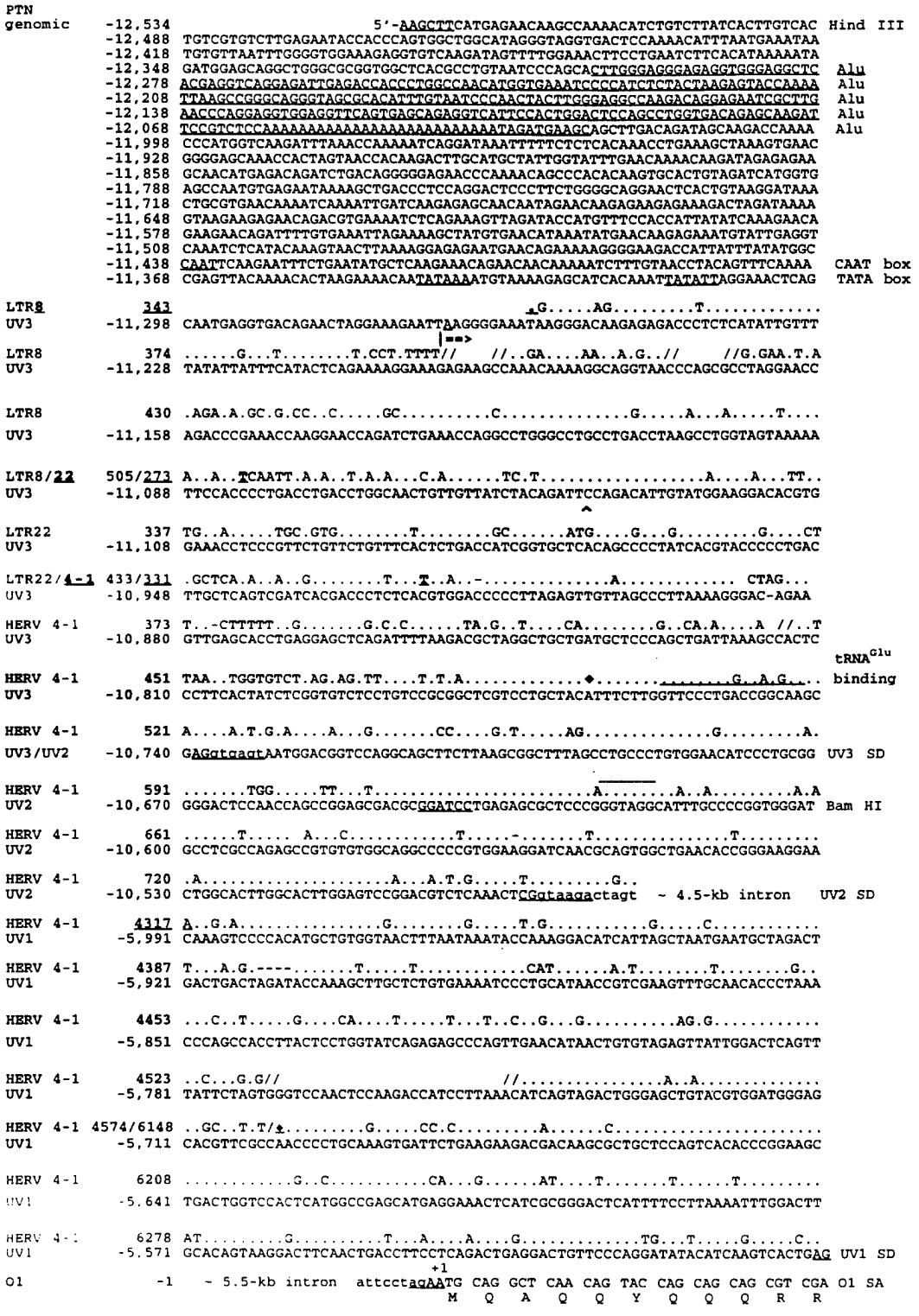
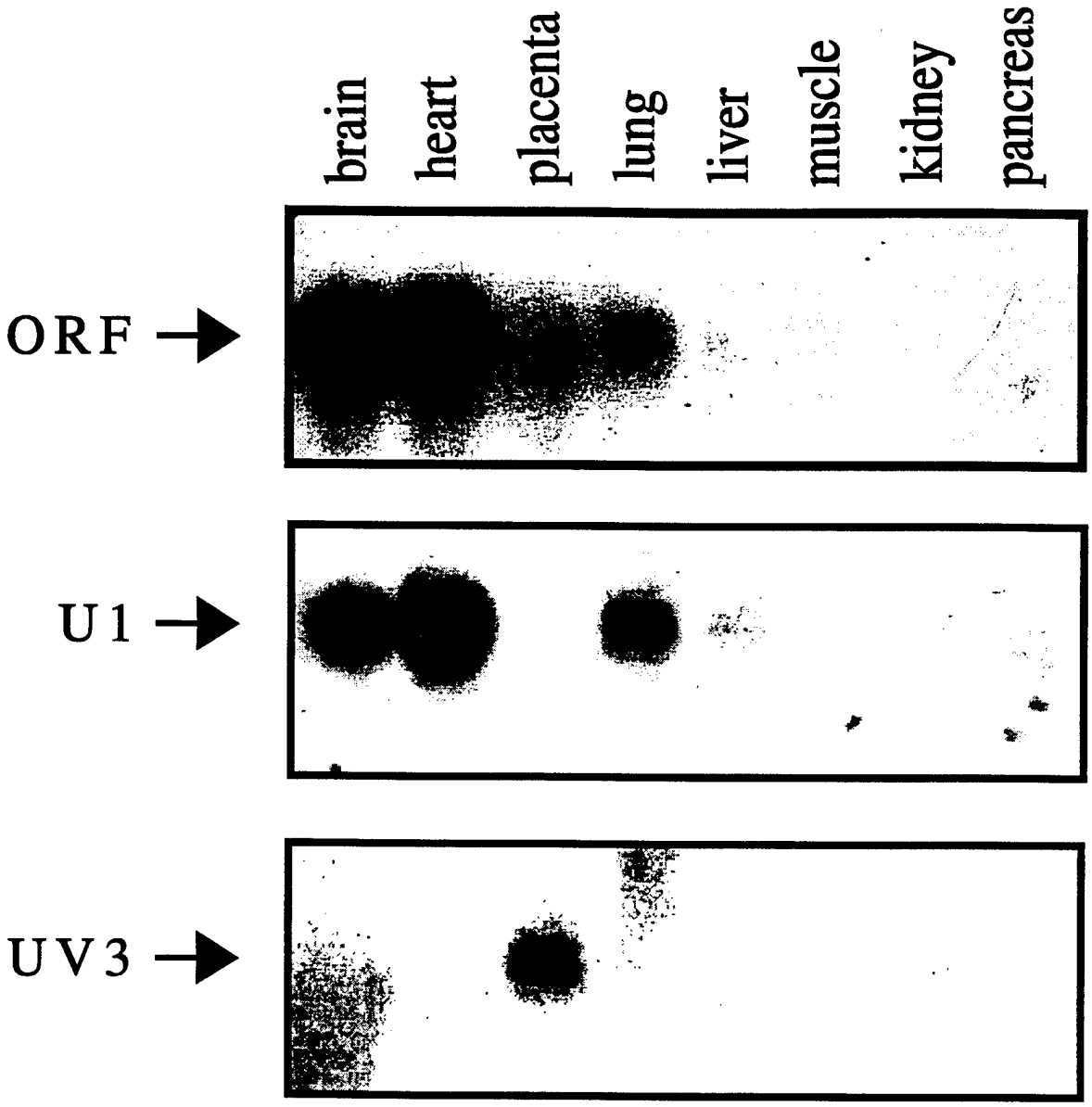


Fig. 1C. Northern blot analysis of mRNA from various human tissues using exon-specific probes.The position of the probes is indicated in Fig. 1A.



2. Promoter activity of the retroviral insertion in the human PTN gene:

Primer extension with primers targeted within the most 5' UV3 exon of the HERV insert mapped the start site of the HERV-PTN transcripts to an A residue 39 nt downstream of putative CAAT and TATA boxes (see Fig. 1B). Presence of these elements at the transcription start site suggested that the HERV insertion might have generated an additional promoter in the intron immediately upstream of the coding region of the human PTN gene. To investigate whether this putative promoter was responsible for the trophoblast-specific expression of HERV-PTN, we performed transient transfection assays with promoter-reporter constructs. Upstream of a luciferase reporter gene, we inserted a genomic fragment that starts 1.5 kb upstream of the transcription start site, contains the CAAT and TATA boxes, and extends downstream of the start site into exon UV2 (Fig. 1B and 2). Transcriptional activity of the resulting construct was observed in human trophoblast / choriocarcinoma cells (JEG-3 and JAR cells), and only when the HERV insert was oriented as in vivo. Luciferase activity in JEG-3 and JAR cells transfected with the HERV promoter-reporter construct in sense orientation was 50- to 100-fold that of cells transfected with the promoterless vector alone. Deletion of the Alu element did not affect this transcriptional activity (not shown). Only background activity was detected in PTN-positive as well as in PTN-negative cells (Fig. 2). We conclude from these data that the HERV insertion in the human PTN gene generates a functional promoter that confers tissue-specific expression.

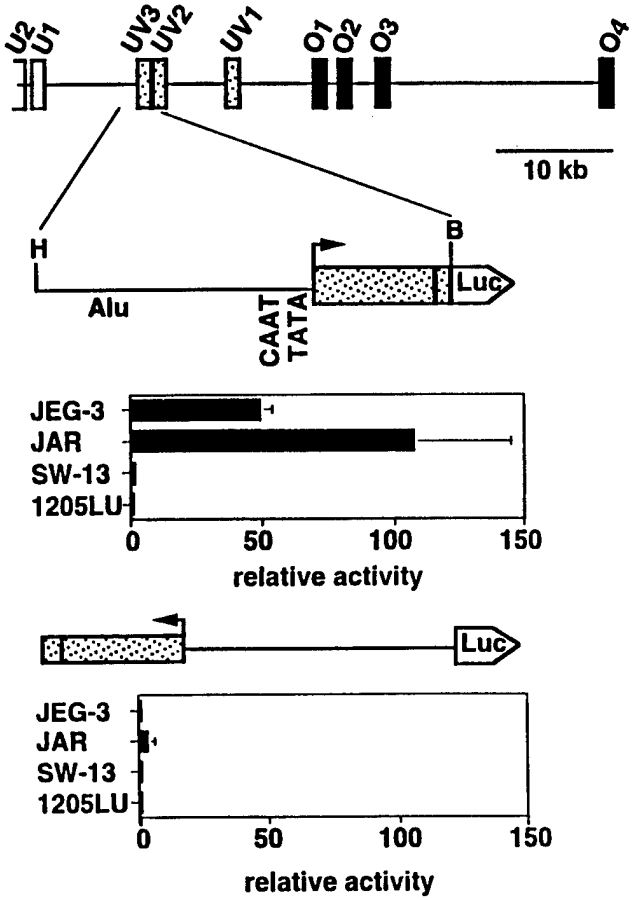


Fig. 2. Promoter activity of the HERV insert. **(legend continued next page)**

-continued legend Fig. 2-
Transcriptional activity of a HindIII / BamHI (H,B) genomic fragment (see Fig. 1B) inserted into the promoterless pXP-1 reporter vector [15] in sense and antisense orientation. Data are means ±SD of triplicate determinations and are representative of at least two independent transient transfection experiments for each cell line. Luciferase activity, normalized to the protein content, is expressed relative to that obtained with the pXP-1 vector. JEG-3 and JAR are different from SW-13 and 1205LU with $p<0.01$.

3. Steroid hormone sensitivity of the retroviral promoter.
A shorter fragment of the HERV promoter was utilized for preliminary studies of hormone sensitivity (see Fig. 3). The intrinsic activity of the fragment alone was significantly higher than the negative control. This is most likely due to the endogenous production of progesterone by the trophoblast cells utilized. Furthermore, the progesterone agonist R5020 stimulated transcriptional activity of this fragment even higher.

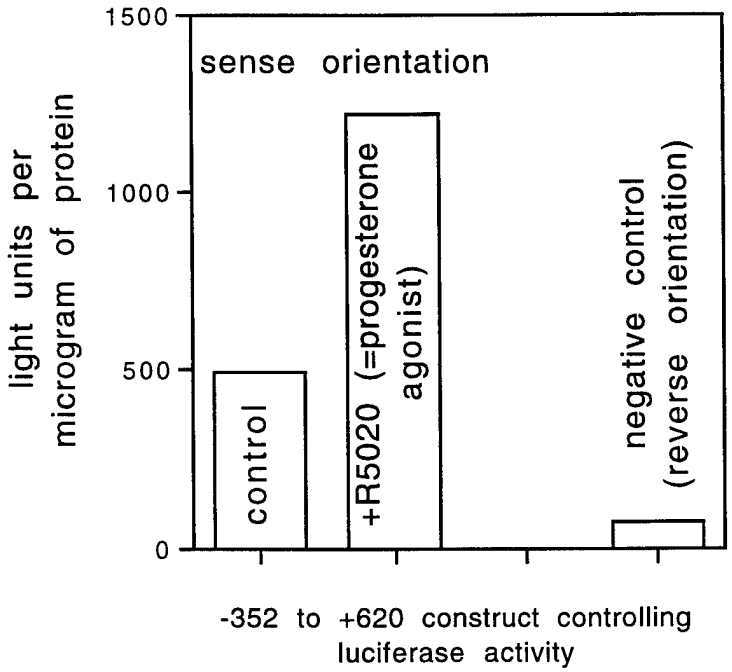


Fig. 3. Effect of a progesterone agonist on an HERV-PTN promoter fragment. A fragment covering region -352 to +620 relative to the transcription start site (see Fig. 1B, above) was cloned upstream of luciferase in the pXP-1 vector and tested for sensitivity to the progesterone agonist R5020 in transient transfection assays in JEG-3 cells. A reverse-oriented construct was used as a negative background control. Mean ±SD is shown (The errors are smaller than 2%). The treatment effect was different from control with a p-value <0.05. Positive control was different from negative control $p<0.01$.

Methods

Gene Structure Analysis.

Non-overlapping phagemid P1 clones (Genome Systems, St. Louis, MI) were used to complete the structural analysis of the 5'untranslated region of the human PTN gene reported earlier [3,14]. P1 clones containing the upstream untranslated exon U1 (P203) or the first exon of the ORF, O1 (P2258), were obtained by PCR screening with specific primers. Long-range PCR (ExpandTM long template PCR; Boehringer Mannheim) with P1 clone 2258 (containing O1) or with human genomic DNA and subcloning and sequencing of inserts was used to compile the structure of the human PTN gene. The nucleic acid sequence of the inserted promoter region and of HERV-derived exons UV3 and in part of UV2 were obtained from a genomic DNA fragment subcloned from a BamHI restriction library of P1 clone P2258 (containing O1) which was screened for positive clones with a UV3-specific probe. Furthermore, the 5'RACE PCR products of placenta cDNA were sequenced for comparison and to complement data from the genomic cloning.

5'RACE PCR

(5'RACE PCR = 5'rapid amplification of complementary DNA ends polymerase chain reaction). Two 5'RACE cDNA libraries generated from human placenta and human brain mRNA (Clontech, Palo Alto, CA) served to generate PCR fragments of the 5'ends of different PTN cDNAs. Nested primers derived from exon O1 were used as 3' primers in the PCR reaction and an antisense oligonucleotide to the anchor sequence was used as a 5'primer. PCR fragments were subcloned into TA-cloning vectors (Invitrogen) and inserts were sequenced.

Mapping of the Transcription Start Site by Primer Extension.

The AMV Reverse Transcriptase (RT) Primer Extension System (Promega, Madison, WI) was used with polyA+ RNA (9 µg) or total RNA (50 µg) as a template. RNA from JEG-3 and JAR choriocarcinoma cells (PTN-positive) or SW-13 cells (PTN negative) was incubated with two UV3-specific, nested primers designed to hybridize to the sequence stretch between -11154 / -11170 and -11168 / -11184 respectively (see Fig. 1B). After denaturation of the RNA for 30 minutes at 65°C, primer hybridization was run for one hour at 52°C followed by a one hour incubation at 42°C with AMV-RT. The samples were then heated for 10 minutes at 90°C in formamide loading buffer and analyzed on a 6 % sequencing gel. Sequencing reactions with each of the primers were used to read the position of the extended product.

Transcriptional Activity.

A 1.9 kb HindIII/BamHI (H,B) genomic fragment from P1 clone P2258 was used for these studies. This fragment starts upstream of the Alu region (-12,534), contains the TATA box and transcription start site of the HERV-PTN fusion transcripts and ends in exon UV2 (-10,640) (see Fig. 1). The fragment was cloned in both orientations into the pXP-1 promoterless luciferase reporter gene vector [15] and then used in transient transfection assays in different cell lines. For this, cells were plated overnight at 60% to 70 % confluence in 6 well plates and then transfected in Optimem (Life Technologies) with 1 µg of DNA per well using 7 µl of Lipofectamine (Life Technologies) for the JEG-3, JAR and SW-13 cells and 2.5 µl of Transfectam (Pharmacia) for the 1205LU cells. After 5 hours, transfection media was replaced by fresh culture medium and the cells were incubated for another 24 to 36 h. Thereafter cells were harvested, washed and lysed in 0.25 M Tris-HCl buffer pH 7.8, freeze-thawed three times and 5 to 20 µl of the lysate were mixed with 350 µl of 0.1 M KPO4, 15 mM MgCl2, 5 mM ATP at pH 7.8 and assayed for luciferase activity using 1 mM D-luciferin as a substrate. The promoter activity is shown as fold induction

relative to the parent vector pXP-1. A CMV-drive luciferase expression vector was used to control for transfection efficacy.

Northern Blot.

Total RNA from cell lines or tissues was isolated with the RNA STAT-60 method (Tel Test Inc.; Friendswood, TX), separated and blotted as reported earlier [2]. In addition, a human multiple tissue Northern blot (Clontech) was used. PTN cDNA probes specific for the ORF [2] or 5'untranslated exon U1 (287 nt fragment) or HERV-derived exon UV3 (257 nt fragment) were hybridized, washed and autoradiographed for 48 hours as described [2]. After exposure, blots were stripped and reprobed. GAPDH was used as a loading control.

Problems & solutions and Next steps:

Analysis of the transcriptional regulation of PTN by hormones using the newly discovered promoter in the human gene is ongoing. In particular, we hope to define next, where regulatory elements are located and what defines response in certain contexts. For this experiments with different hormone-dependent and independent breast cancer cell lines have been planned.

Specific Aim (2):
To study the effect of expression of PTN on the malignant phenotype of PTN-negative breast cancer cells.

Background:
Hormones and growth factors define the capacity of human breast cancer to grow and ultimately to metastasize. One of the essential requirements for the development of breast cancer are circulating steroid hormones and one of the most widely used drug therapies of breast cancer with the anti-estrogen tamoxifen is based on this fact. Furthermore, growth factor gene expression can supplement for hormone stimulation and thus contribute to hormone-independent cancer growth as well as to resistance to anti-hormone therapy (reviewed e.g. in [8]).

Work accomplished:

1. Transfection of PTN into PTN-negative breast cancer cells:
We have transfected PTN-negative, estrogen-dependent MCF-7 wild-type and T-47D wild-type human breast cancer cells with an expression construct for PTN (see [2] and have generated a series of different cell lines (mass-transfected and some clonal cell lines) expressing PTN. We have tested the cells *in vitro* for their proliferation and colony forming abilities as well as for expression of PTN mRNA and the secretion of protein.
A wide range of expression levels of PTN was achieved in different MCF-7-derived and T-47D-derived cell lines. No gross difference in the *in vitro* phenotype of the cells was observed. No significantly different proliferation on plastic surface or colony forming ability was found. Based on the current data we conclude that PTN is not utilized by T-47D or by MCF-7 cells as an autocrine growth factor. However, these *in vitro* experiments need to be expanded with hormone and anti-hormone addition to challenge the cells.

Currently, the further *in vitro* analysis of these cell lines is underway and we plan to initiate animal tumor growth studies after completion of the *in vitro* analysis.

2. Animal studies:
We have not initiated animal studies.

Next steps:
We plan to complete the *in vitro* analysis of the growth characteristics and hormone effects on transfected cells.

Specific Aim (3):
The function of the different domains of the PTN protein

Background:
The secreted PTN protein contains two distinct cysteine-rich domains (on two separate exons) that contains three and two disulfide bridges respectively. Disulfide bridge formation is required for biological activity of the protein. We hypothesize that defined mutations will generate a protein that can still bind to the receptor but will fail to activate the receptor and can thus serve as an antagonist.

Work accomplished earlier:
We have generated point mutant PTNs that have the N-terminal or the C-terminal cysteine changed to a serine and thus disrupted disulfide bridge formation. We tested the effects of the mutant proteins in transfection assays using expression vectors for the mutant and for wild-type PTN in PTN-responsive SW-13 cells as indicator cells of activity.
To our astonishment we found that the N- or C-terminal cysteine mutations affect the activity in transient transfection assays only very little. Furthermore, the amount of protein secreted is not decreased suggesting that the mutant proteins are as stable as the wild-type protein. We conclude from this finding that the N-terminal and the C-terminal disulfide bridge are not essential for stability and activity of the protein when only one of them is destroyed.

Recent work in this report cycle:
To assess the activity of the differently mutated forms of PTN more easily and more directly, we set up a short-term assay that would detect the induction of phosphorylation of proteins in the PTN signal-transduction pathway. We characterized by immunoprecipitation and by Western-blotting with anti-phosphotyrosine antibodies a 190 kDa protein as well as MAP-Kinase proteins that are tyrosine-phosphorylated within 10 minutes after stimulation of PTN-responsive cells in culture. Currently, this assay is used to assess the activity of different preparations of the mutated protein.

Problems & solutions and Next steps:
Cross-competition between wild-type and mutated PTN protein are planned to work out whether at this level of the earliest signal transduction we could easily and quickly screen for inhibitory mutated protein.

Specific Aim 4:
To inhibit production of PTN

Background

We planned to use three independent approaches to target PTN mRNA and thus reduce the amount of PTN produced by PTN-positive breast cancer cells:

1. antisense oligonucleotides
2. antisense constructs
3. ribozyme constructs

Data from the first two approaches were shown in the last report. Here we present work accomplished now that demonstrates that PTN drives tumor invasion, angiogenesis and metastasis. This proof was achieved by depleting tumor cells of their endogenously produced PTN using targeting with different ribozyme constructs.

Work accomplished

1. Biological function of the PTN gene product generated due to the retroviral promoter (=HERV-PTN mRNA).

Cells in which **only** the retroviral promoter was actively driving expression of PTN (Fig. 4A) were used to screen for PTN protein secretion (Fig. 4B) and then for its potential biological role in tumor cell growth in vitro as well as invasion, growth and angiogenesis in animal models.

Depletion of HERV-PTN with ribozymes. We investigated the biological significance of the expression of HERV-PTN using JEG-3 choriocarcinoma cells (see Fig. 4) as a model system. Tumor growth of these cells in experimental animals mimics the highly invasive and angiogenic growth phenotype of the normal human trophoblast [16] as well as clinical choriocarcinoma and we hypothesized that one of the contributing factors to this phenotype could be the expression of HERV-PTN. To address this hypothesis, we examined the effects of reducing the abundance of HERV-PTN transcripts in JEG-3 cells by stable expression of a PTN-targeted ribozyme [17]. A vector (pTET/Rz261) with high transcriptional activity in these cells (see "Methods") was used to express the ribozyme.

Northern blot analysis revealed that ribozyme expression reduced the amount of HERV-PTN mRNA in JEG-3 cells to background levels (Fig. 5A). No difference in the proliferation rate of PTN-depleted versus control cells was apparent in vitro (Fig. 5B), suggesting that the cells do not require PTN as an autocrine growth factor even though they secrete the protein in a biologically active form (see above). However, a marked difference in the growth phenotype of PTN-depleted versus control cells was observed after xenografting tumor cells into athymic nude mice. In an initial study, we implanted the tumor cells into their "natural", intra-abdominal environment to observe their orthotopic growth behavior. The control cells formed large tumor masses that invaded the abdominal organs within 2 to 3 weeks, whereas only a few, small seedings of PTN-depleted tumor cells were detected in the abdomen at the end of the study (n=5 and n=4 animals respectively; Fig. 5C). Parallel results were obtained after subcutaneous injection of tumor cells. In contrast to control cells, which grew rapidly into highly angiogenic tumors (n=7; Fig. 5D), no tumor growth was observed with PTN-depleted cells (n=9; Fig. 5D). The data were highly statistically significant ($p<0.001$).

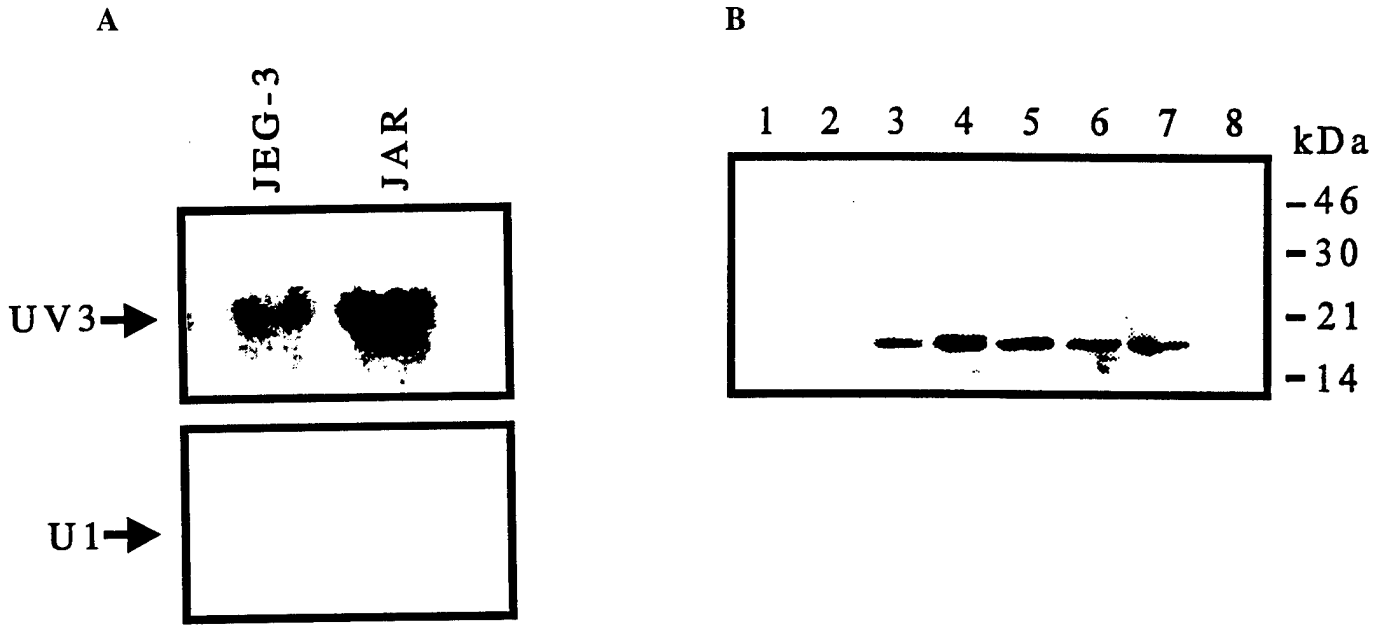


Fig. 4. Northern blot for PTN mRNA with exon-specific probes **(A)** -as in Fig. 1C- and Western blot for PTN protein [1] in the supernatants of JEG-3 cells **(B)**

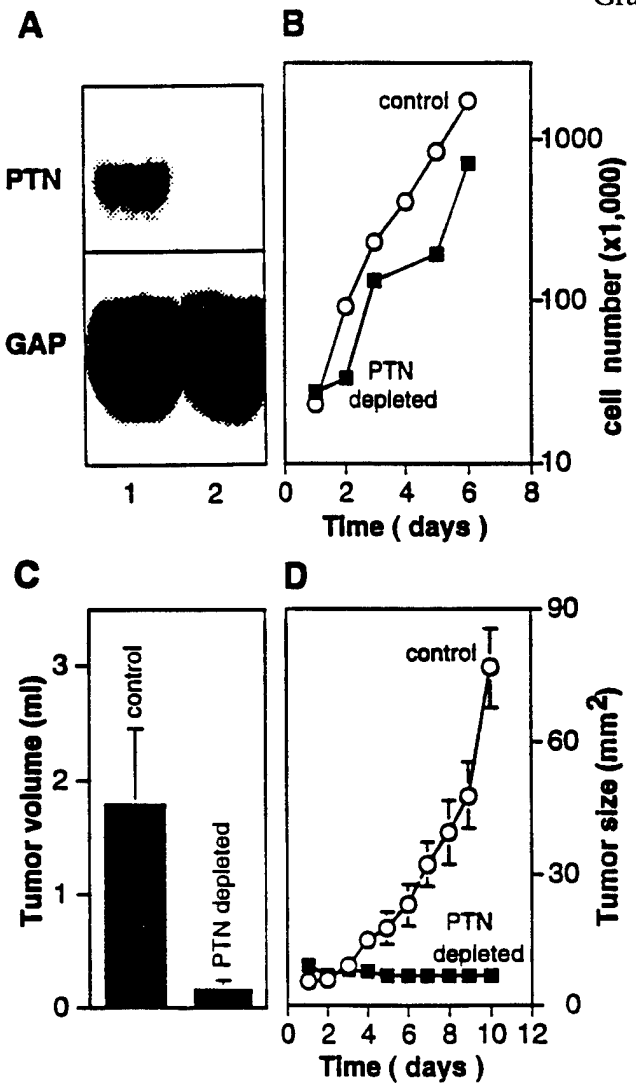


Fig. 5. Effect of the depletion of HERV-PTN mRNA from JEG-3 cells.
(A) Northern blot analysis of total RNA from control JEG-3 cells (lane 1), and JEG-3 cells expressing a ribozyme targeted to PTN mRNA (lane 2).
(B) Proliferation in vitro of control and PTN mRNA-depleted JEG-3 cells.
(C) Intraperitoneal growth of two million tumor cells (mean ± SD)
(D) Growth curves of subcutaneous tumors in athymic nude mice. Two million control or PTN mRNA depleted JEG-3 cells were inoculated subcutaneously into athymic nude mice. Data represent mean tumor sizes ±SD. Control versus PTN-depleted tumor growth $p<0.001$.

Macroscopic inspection as well as microscopic analysis after H&E staining of tumor sections from Fig. 5 showed reduced blood vessel growth as well as reduced invasion of normal tissues.

We conclude that the HERV-driven PTN transcript can be an essential and rate-limiting factor for tumor growth, invasion, and angiogenesis *in vivo*. Since the PTN protein is the same irrespective of the promoter driving gene transcription (see Fig. 1B and Fig. 4B) this biological function should be the same for the protein produced by different types of tumor cells.

2. "Gene-dose-response" of PTN for tumor angiogenesis and metastasis

To assess whether PTN is a rate-limiting factor for tumor growth in vivo, angiogenesis and metastasis, we took advantage of a human cell line that produces high levels of PTN mRNA, grows into subcutaneous tumors in mice and metastasizes from the subcutaneous site as hematogenous metastases into the lung. These metastases can be observed 6 to 9 weeks after inoculation of the primary tumor cells. The cell line (named 1205LU) was generated by Dr. M. Herlyn (Wistar Institute, Philadelphia, PA) who kindly provided them to us.

By transfection with ribozyme expression vectors, we generated a panel of derivative clonal and mass-transfected 1205LU cells that expressed different residual levels of PTN mRNA and protein. We separated the cell lines accordingly into high (= close to control levels), medium (= 60 to 75% of control) and low (= <30% of control) PTN expressors and analyzed the in vitro and animal tumor growth data accordingly.

In vitro growth on plastic surface as well as colony formation in soft agar by the 1205LU cells expressing different levels of PTN was unaffected by the changes in PTN levels (not shown).

However, subcutaneous tumor growth (Fig. 6a and b) was slowed dramatically even in the medium group that had a less than 50% reduction of endogenous PTN. The low group took several months to catch up in their growth with the control group. A direct relationship between tumor growth and residual "gene dose" of PTN was observed (see Fig. 6b)

Angiogenesis was quantitated in the subcutaneous tumors after they had been allowed to grow to the same size (i.e. after different time periods depending on the subgroup). A significant reduction of the number of blood vessels per sqmm due to the reduction of PTN in the tumor cells was observed. This effect was "gene-dose-dependent".

Finally, upon analysis of macroscopic and microscopic sections of the lungs, in all of the animals carrying the PTN-depleted tumors, no macroscopic metastases were seen. After microscopic metastases were tested for by serial sections of the lungs, each animal with any sign of a metastasis was counted as positive and a significant reduction in the incidence of animals with metastasis was found (Fig. 6d).

We conclude from this data that PTN can be a rate-limiting factor for tumor growth, angiogenesis and metastasis. No threshold effect with regard to a minimum level of reduction of PTN was observed.

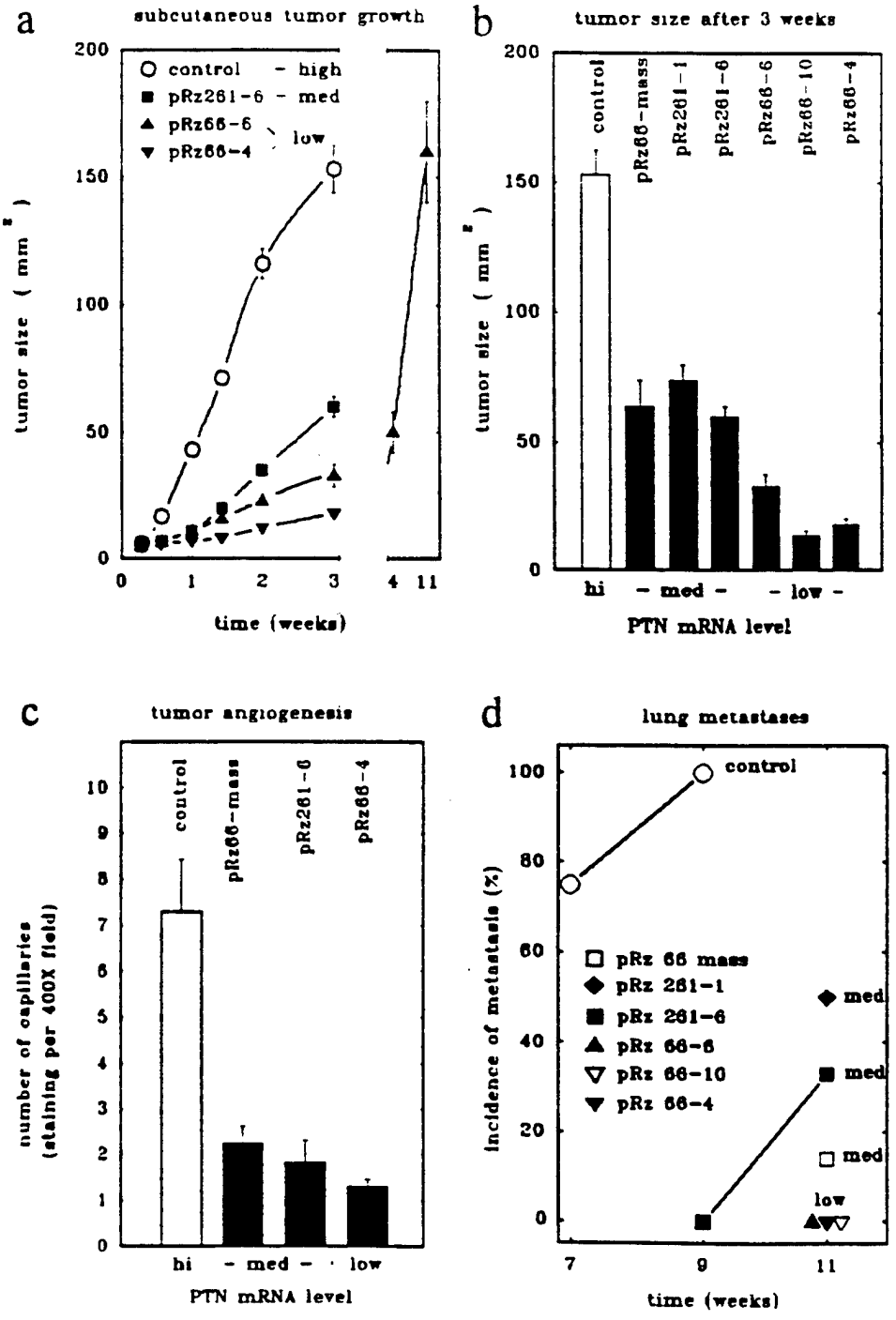


Fig. 6. Subcutaneous tumor growth (a,b), angiogenesis (c) and lung metastasis (d) in nude mice of 1205LU melanoma cells producing different levels of PTN mRNA.

a and b, complete growth curves of subcutaneous tumors from representative cell lines (a) and tumor sizes of all cell lines after three weeks (b) are shown. The cell lines producing different

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- continuation of legend to Fig. 6. -

levels of PTN mRNA were injected subcutaneously into nude mice (n = 5 to 12 animals per group) at 1 million cells per injection site and two sites per animal. Tumor growth of the "medium" versus control and the "low" versus control group was different at $p<0.01$ each.

c, angiogenesis in subcutaneous tumors grown from representative groups of the melanoma cells expressing different levels of PTN mRNA was quantitated. Capillaries in tumors were highlighted by staining for PECAM (=CD31). In three representative tumors from each group the average number of capillaries in eight high power fields (400X) was counted. The number of capillaries/field ± standard error of the counts of three blinded investigators are shown ("medium" versus control $p<0.01$; "low" versus control $p<0.002$).

d, quantitation of lung metastases. Subcutaneous tumors were resected after 3 weeks (controls), 11 weeks (pRz66-6) or when they reached at least 50 mm2. Lungs were examined macroscopically or after H&E staining. Animals with tumors from high level PTN producing cells showed macroscopically visible metastases whereas metastases in the others groups were only detectable after microscopic inspection of lung sections. Animals with any detectable metastases were scored positive and the relative incidence of animals with lung metastases is shown. $p<0.01$ for the incidence of metastasis in the "medium" PTN-reduced versus control and $p<0.002$ for the "low" versus control group.

Problems & solutions and next steps

Transfections of PTN-positive human breast cancer cells with PTN-targeted ribozymes are planned as a next round of experiments.

Methods:

Depletion of PTN mRNA using Ribozyme Targeting.

The PTN-targeted ribozyme Rz261 [17] was expressed under the control of the tTA / heptameric operator binding site and a CMV minimal promoter [18]. For this purpose, the major portion of the luciferase gene and the SV40 polyadenylation site in the pUHC13-3 plasmid [18] were deleted by HindIII / HpaI cut and replaced with the Rz261 / bovine growth hormone polyadenylation HindIII / PvuII fragment from the pRc/Rz261 expression vector [17]. The remaining luciferase start codon was replaced by a SalI / ClaI / HindIII cassette to yield the construct pTET/Rz261. This ribozyme is designed to cleave PTN mRNA 3' of nucleotide 261 of the ORF [17]. In JEG-3 cells the ribozyme expression vector (pTET/Rz261 , 0.5 μg), was co-transfected with the tTA expression vector (pUHG15-1 [18], 0.5 μg) and pRc/CMV (0.1 μg) to provide G-418 resistance. After selection for stable integrants in the presence of 1 mg/ml of G-418, the cells were tested for PTN expression by Northern analysis.

CONCLUSIONS

We discovered a novel retrovirally-derived promoter in the human PTN gene (published in reference [6]) and have now embarked on the hormone-regulatory elements in this promoter. Furthermore, we have expanded our ribozyme-targeting studies to demonstrate the role of PTN for tumor growth, invasion, angiogenesis and metastasis (published in reference [6] and [7]). Finally, signal transduction studies will be utilized to assess the functionality of mutated PTN protein as a stimulator or inhibitor.

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