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13. ABSTRACT *(Maximum 200 Words)*

Angiogenesis, the formation of new blood vessels from pre-existing ones, plays an important role in breast cancer growth and metastasis. The rational design of novel drugs targeting angiogenesis requires an understanding of the signaling pathways that mediate angiogenesis, from growth factors through their receptors to the activation of intracellular signaling cascades. The pleiotrophin signaling pathway is known to be important in angiogenesis and breast cancer growth, but the exact mechanisms of pleiotrophin signaling remain undefined. We have recently identified a novel cell-surface receptor (PTNR) capable of mediating pleiotrophin's mitogenic and angiogenic activities. Here we demonstrate the biologic relevance of pleiotrophin signaling through PTNR. By targeting PTNR in appropriate cell lines through the use of hammerhead ribozymes, we can reduce the endogenous levels of PTNR mRNA as demonstrated by a PTNR-specific RNase protection assay. In addition, signaling through PTNR can also be disrupted by overexpressing dominant-negative PTNR constructs in appropriate cell lines. The phenotype resulting from these two independent approaches can be demonstrated by analysis of the ability of altered tumor cells to form colonies in soft agar. A final goal is to determine the molecular interaction between pleiotrophin and PTNR by mutational analysis of the ligand-binding domain of PTNR.

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INTRODUCTION

Angiogenesis is vital for the growth of solid tumors, by providing essential nutrients, and for tumor metastasis, by providing access to the general circulation. Without angiogenesis solid tumors could not grow beyond a few millimeters in size[1]. Signaling pathways utilized by angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have already been identified and continue to be defined. However, the signaling pathways activated by the angiogenic factor pleiotrophin remain unknown, largely because a receptor capable of mediating pleiotrophin's angiogenic activity has not yet been convincingly identified. We have recently identified the anaplastic lymphoma kinase (ALK) as a novel receptor for pleiotrophin (PTNR)[2]. Here we report on our studies into the biological relevance of pleiotrophin signaling through PTNR.

BODY

The research accomplishments described herein cover Task 1 in the approved Statement of Work in the original grant application. The goal of Task 1 was to use PTNR-targeting ribozymes to determine the role of pleiotrophin signaling through PTNR in tumor growth. The first step towards this goal was to develop a quantitative assay capable of measuring levels of PTNR mRNA in human cell lines. A number of cell lines had already been shown to express PTNR mRNA by reverse transcription polymerase chain reaction (RT-PCR), but in order to show ribozyme efficacy a quantitative assay was necessary. Based on cDNA sequence information, a riboprobe was designed that was capable of hybridizing to a stretch of the PTNR mRNA between nucleotides 1104 to 1341 (Figure 1*a*). Using this riboprobe, RNase protection

analysis was performed on cell lines known to be PTNR-positive by RT-PCR (Figure 1*b*). Only the human glioblastoma cell line U87MG expressed enough PTNR mRNA to allow detection by this assay. Screening a panel of glioblastoma cell lines revealed three of seven human cell lines were positive for PTNR mRNA by RNase protection. The U87MG cell line was used for further studies into pleiotrophin/PTNR signaling as described in Task 1 because ribozyme-mediated reduction of their endogenous expression of PTNR mRNA could be more easily determined than the reduction of PTNR mRNA in a lower-expressing cell line. Studies are currently being undertaken to develop a quantitative RT-PCR protocol for those cell lines in which PTNR was undetectable by RNase protection.

The second step in Task 1 was to show depletion of endogenous PTNR in cells stably transfected with PTNR-targeting ribozymes. Two distinct ribozyme constructs were utilized: Rz1 and Rz12, targeting PTNR mRNA at nucleotides 652 and 4739, respectively (Figure 2*a*). These ribozyme constructs were cloned into a CMV-driven mammalian expression vector (pRC/CMV) and transfected into wildtype U87MG cells. Stable clones were selected by growth in G418-containing medium. RNA was then harvested from these stable clones and analyzed by RNase protection for PTNR. Nine clones of Rz1-transfected cells and eleven clones of Rz12-transfected cells were analyzed (Figure 2*b* and Figure 2*c*, respectively). These two panels of cells displayed a range of residual PTNR mRNA levels, probably due to the random nature of construct integration and the resulting variations in ribozyme expression. From these two panels, a third panel of clones was analyzed, along with the control empty vector mass-transfected cells (designated "pRC/CMV") (Figure 2*d*). Phosphoimager analysis of this RNase protection experiment reveals a panel of cells with a range of residual PTNR mRNA (Figure 2*e*). This

allowed us to undertake "gene dose-response" experiments into the relevance of pleiotrophin signaling through PTNR in the context of cells with ranges of residual PTNR.

The final step of Task 1 was to determine the biological relevance of PTNR depletion by analysis of colony formation in soft agar. A single cell suspension of each cell was plated in agar and allowed to grow for two weeks, at which time those cells capable of growing in the absence of contact with a basement membrane would have formed colonies that could be detected by our image analysis equipment. This experiment was undertaken with the panel of PTNR-depleted cell lines both in the presence and absence of serum-containing medium. While these cells formed more colonies in the presence of serum, there was no difference in colony formation that correlated with levels of residual PTNR mRNA (Figure 3*a* and Figure 3*b*). To determine if the addition of pleiotrophin might differentially affect the cells with the highest (pRC/CMV) and lowest (Rz1-2 and Rz1-7) levels of PTNR exogenous pleiotrophin was added to these cells in a soft agar assay. The addition of exogenous pleiotrophin had no differential effect on colony formation of these cells (Figure 3*c*).

Figure 1 PTNR mRNA can be detected by RNase protection in human glioblastoma cell lines. ***a***, PTNR riboprobe for RNase protection experiments contains a 237 nucleotide stretch complementary to the PTNR cDNA segment from 1104 to 1341. ***b***, Detection of PTNR mRNA by RNase protection in U87MG cells, but not in other cell lines shown to be PTNR positive by RT-PCR. GAPDH is used as a loading control. ***c***, Detection of PTNR mRNA in human glial cell lines by RNase protection. Three of seven glial cell lines (U87MG, U118MG and U138MG) are positive for PTNR mRNA. The loading control, 36B4, reveals the integrity of RNA in all samples.

FIGURE 1

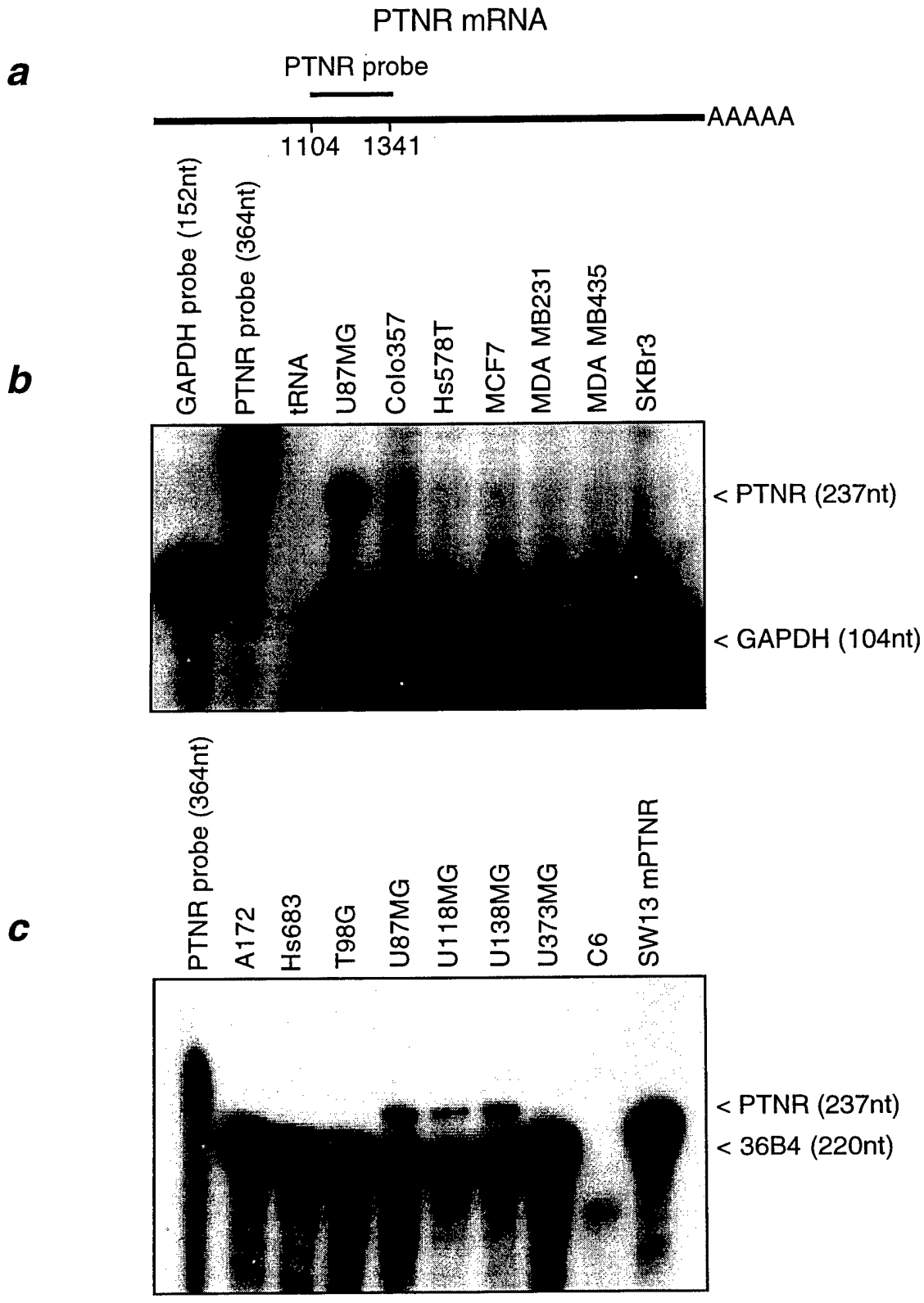

a
PTNR mRNA
PTNR probe
AAAAA
1104
1341
b
GAPDH probe (152nt)
PTNR probe (364nt)
tRNA
U87MG
Colo357
Hs578T
MCF7
MDA MB231
MDA MB435
SKBr3
< PTNR (237nt)
< GAPDH (104nt)
c
PTNR probe (364nt)
A172
Hs683
T98G
U87MG
U118MG
U138MG
U373MG
C6
SW13 mPTNR
< PTNR (237nt)
< 36B4 (220nt)

Figure 2 Ribozyme-targeting of PTNR depletes endogenous PTNR mRNA in a panel of U87MG clones. ***a***, Two distinct ribozyme constructs are utilized: Rz1 and Rz12, which cleave PTNR mRNA at nucleotides 652 and 4739, respectively. ***b,c*** RNase protection assays of 60 μg of total RNA from U87MG clones transfected with the ribozyme constructs Rz1 (***b***) or Rz12 (***c***). Equal loading of RNA is insured by the GAPDH signal. ***d***, RNase protection assay of final panel of U87MG ribozyme clones and empty vector (pRC/CMV) mass-transfected U87MG cells. ***e***, Quantification of RNase protection assay by phosphoimager analysis; PTNR/GAPDH signals are represented relative to pRC/CMV control.

FIGURE 2

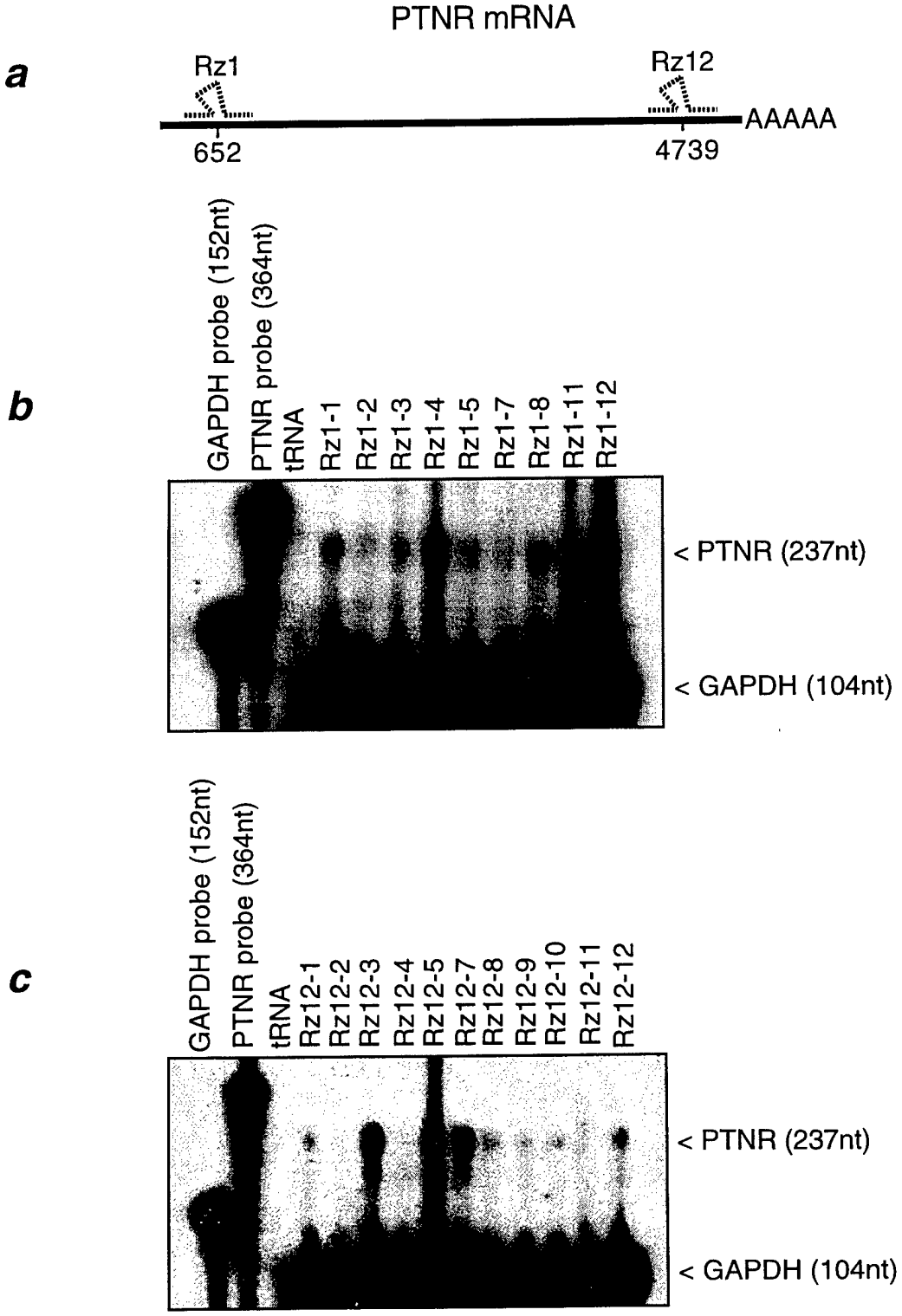
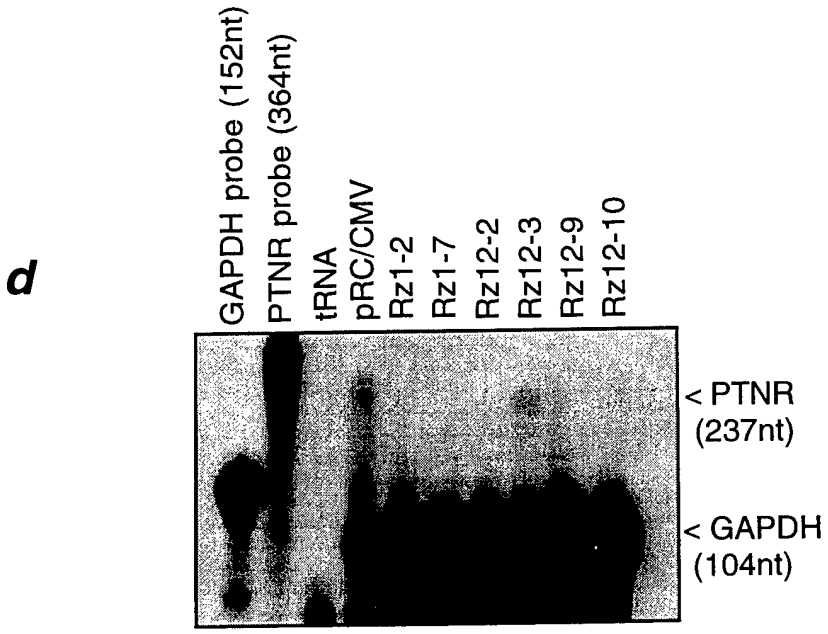

a
PTNR mRNA
Rz1
Rz12
AAAAA
652
4739
b
GAPDH probe (152nt)
PTNR probe (364nt)
tRNA
Rz1-1
Rz1-2
Rz1-3
Rz1-4
Rz1-5
Rz1-7
Rz1-8
Rz1-11
Rz1-12
< PTNR (237nt)
< GAPDH (104nt)
c
GAPDH probe (152nt)
PTNR probe (364nt)
tRNA
Rz12-1
Rz12-2
Rz12-3
Rz12-4
Rz12-5
Rz12-7
Rz12-8
Rz12-9
Rz12-10
Rz12-11
Rz12-12
< PTNR (237nt)
< GAPDH (104nt)

FIGURE 2


d
GAPDH probe (152nt)
PTNR probe (364nt)
tRNA
pRC/CMV
Rz1-2
Rz1-7
Rz12-2
Rz12-3
Rz12-9
Rz12-10
< PTNR (237nt)
< GAPDH (104nt)

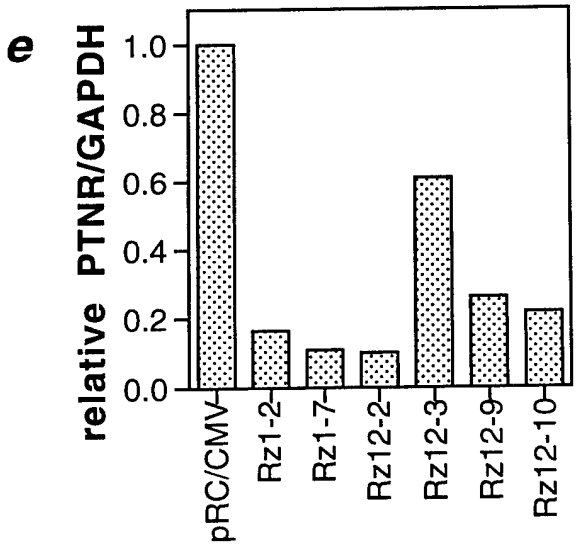

e
relative PTNR/GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
pRC/CMV
Rz1-2
Rz1-7
Rz12-2
Rz12-3
Rz12-9
Rz12-10

Figure 3 Ribozyme-mediated depletion of endogenous PTNR in U87MG cells does not alter colony formation in soft agar. ***a***,***b***, Soft agar colony formation in the presence (***a***) or absence (***b***) of serum of the panel of PTNR-depleted U87MG cells is not significantly different. ***c***, Pleiotrophin treatment does not alter soft agar colony formation of control or PTNR-depleted U87MG cells in serum-free conditions.

FIGURE 3

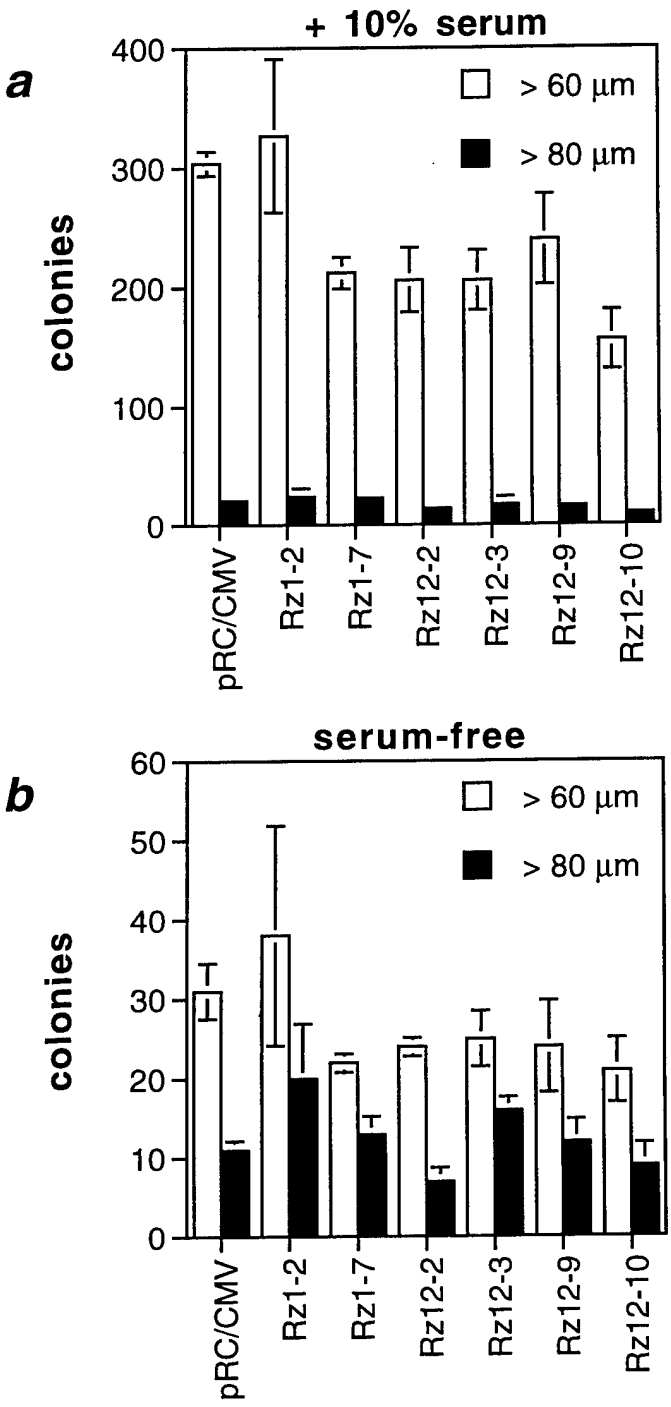
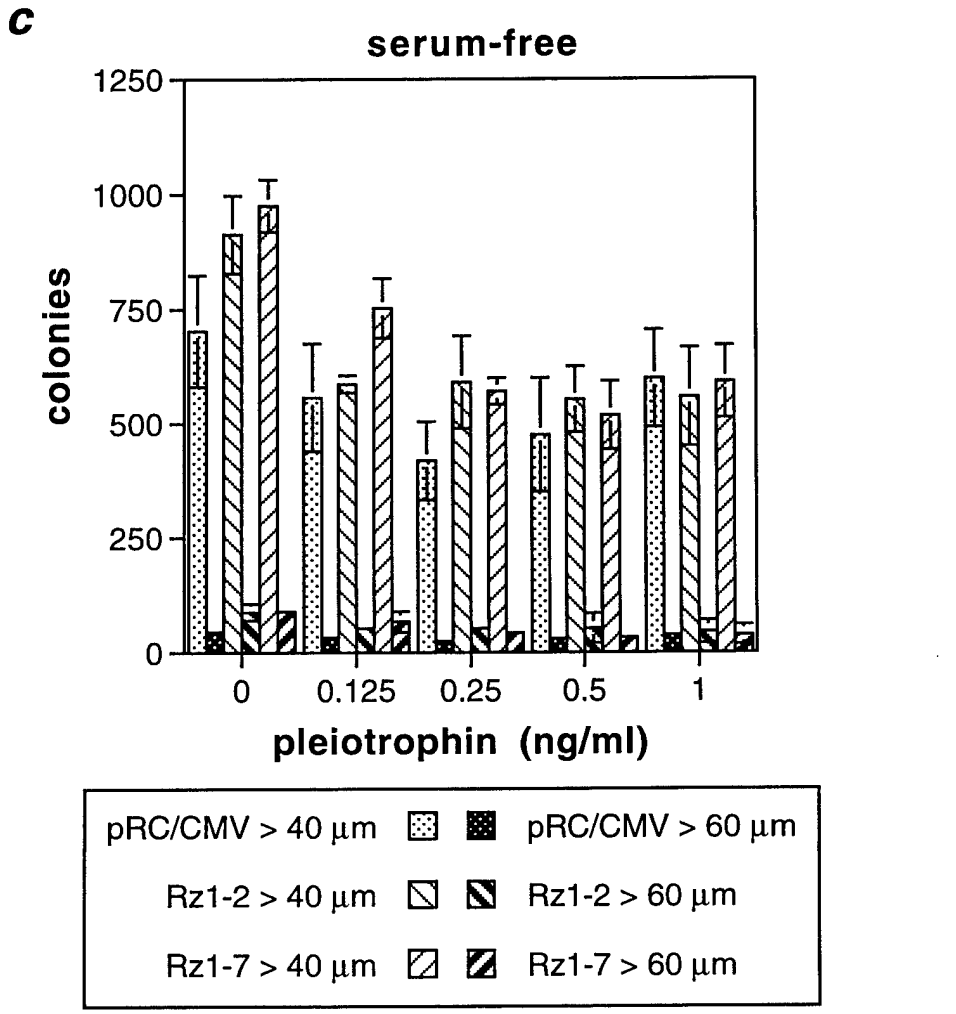

a
+ 10% serum
colonies
400
300
200
100
0
> 60 µm
> 80 µm
pRC/CMV
Rz1-2
Rz1-7
Rz12-2
Rz12-3
Rz12-9
Rz12-10
b
serum-free
colonies
60
50
40
30
20
10
0
> 60 µm
> 80 µm
pRC/CMV
Rz1-2
Rz1-7
Rz12-2
Rz12-3
Rz12-9
Rz12-10

FIGURE 3


c
serum-free
1250
1000
750
500
250
0
colonies
0
0.125
0.25
0.5
1
pleiotrophin (ng/ml)
pRC/CMV > 40 µm
pRC/CMV > 60 µm
Rz1-2 > 40 µm
Rz1-2 > 60 µm
Rz1-7 > 40 µm
Rz1-7 > 60 µm

KEY RESEARCH ACCOMPLISHMENTS

- The baseline level of PTNR mRNA expression in a panel of human cell lines was determined by RNase protection (Figure 1*b* and Figure 1*c*).
- Stable transfectants expressing PTNR-targeting ribozymes were generated and efficacy of ribozyme activity was determined by RNase protection (Figures 2*b-e*).
- Soft agar assays were used to detect changes in phenotype of ribozyme-transfected cells (Figures 3*a-c*).

REPORTABLE OUTCOMES

Doctoral Dissertation:

"Pleiotrophin Signaling Through PTNR in Glioblastoma Multiforme", March 2001.

Abstracts:

Powers, C., Aigner, A., and Wellstein, A. Pleiotrophin signaling through PTNR in glioblastoma multiforme. Fifteenth Annual Student Research Days (2001). 1st Place, Student Division.

Powers, C., Aigner, A., and Wellstein, A. Pleiotrophin signaling through PTNR in glioblastoma multiforme. Second Annual Lombardi Poster Session (2001). 1st Place, Pre-doctoral Division.

Stoica G.E., Aigner, A., **Powers, C.**, List, H.-J., Kuo, A., Bowden, E.T., Riegel, A.T., and Wellstein, A. Pleiotrophin binds and activates a receptor tyrosine kinase. American Association of Cancer Research, New Orleans, LA (2001).

Kuo, A., Stoica, G.E., **Powers, C.**, Bowden, E., Riegel, A.T., and Wellstein, A. Characterization of Pleiotrophin Signal Transduction Through a Receptor Tyrosine Kinase. American Association of Cancer Research, New Orleans, LA (2001).

CONCLUSIONS

While the above described experiments yielded a negative result (i.e. no difference in colony formation in spite of the reduction of endogenous PTNR) they did meet the goals described in Task 1 of the approved grant application. U87MG cells may be a good model system to demonstrate efficacy of ribozyme targeting, however, they do not appear to be an appropriate system to demonstrate the biological effects of such targeting. U87MG cells are known to express a variety of receptor tyrosine kinases in addition to PTNR, such as the epidermal growth factor receptor[3] and the platelet-derived growth factor receptor[4]. Perhaps the presence of these other receptors allows U87MG cells to compensate for the loss of PTNR. Alternatively, pleiotrophin signaling through PTNR in U87MG cells might activate a survival pathway, as opposed to a proliferative pathway, and colony formation in soft agar may not be the best experiment to detect the activity such a pathway. The development of a more sensitive technique than the currently used RNase protection assay for quantitative analysis of PTNR mRNA levels should allow analysis of ribozyme activity in other, more appropriate, cell lines. In addition, overexpression studies of dominant-negative PTNR constructs in other cell lines, as described in Task 2, may be a better approach for determining the relevance of pleiotrophin signaling through PTNR in terms of soft agar colony formation.

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4. Nister, M., et al., *Differential expression of platelet-derived growth factor receptors in human malignant glioma cell lines.* J Biol Chem, 1991. **266**(25): p. 16755-63.