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ORIGINAL ARTICLE

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# Peptides designed from molecular modeling studies of the *ras*-p21 protein induce phenotypic reversion of a pancreatic carcinoma cell line but have no effect on normal pancreatic acinar cell growth

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**Abstract** *Purpose*: From molecular modeling studies we found that two *ras*-p21 peptides, corresponding to p21 residues 35–47 (PNC-7) and 96–110 (PNC-2), selectively block oncogenic (Val 12-p21), but not insulin-activated wild-type, p21-induced oocyte maturation. Our purpose was to determine if these peptides block the growth of mammalian cancer cells but not their normal counterpart cells. *Methods*: Since oncogenic *ras* has been implicated as a causative factor in over 90% of human pancreatic cancers, we have established a normal pancreatic acinar cell line (BMRPA1) and the corresponding *ras*-transformed pancreatic cancer cell line (TUC-3). We treated both cell lines with PNC-7 and PNC-2 and the unrelated negative control peptide, X13, attached to the penetratin sequence that allows membrane penetration and also transfected these cell lines with plasmids encoding all three peptides. *Results*: Treatment of TUC-3 cells with each peptide resulted in their complete phenotypic reversion to the untransformed phenotype as revealed by the lack of tumor formation of these revertant cells implanted in the peritoneal cavities of nude mice. In contrast, treatment with X13-leader resulted in no inhibition of cell growth. Identical results were obtained when TUC-3 cells were transfected with plasmids expressing PNC-2, PNC-7 and X13. None of these peptides affected the normal growth of BMRPA1 cells. *Conclusions*: PNC-2 and PNC-7 peptides induce phenotypic reversion of *ras*-induced pancreatic cancer cells and have no effect on normal pancreatic cell growth. Since the plasmid encoding PNC-2 without penetratin also had the same effect on the TUC-3 cell line, we conclude that the penetratin sequence has no effect on the activity of this peptide. Since X13 attached to penetratin had no effect on TUC-3 cells, the effect is specific for PNC-2 and PNC-7 and further confirms that the effect is not caused by the penetratin sequence. PNC-2- and PNC-7-penetratin may therefore be useful in the treatment of *ras*-induced pancreatic carcinomas.

**Keywords** *ras*-p21 peptides · Molecular modeling · Phenotypic reversion · Pancreatic cancer

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## Introduction

Oncogenic *ras*-p21 protein, but not its wild-type counterpart protein, induces malignant transformation of mammalian cell lines such as NIH 3T3 cells [1] and has been implicated as a major causative factor in a high proportion of human solid tissue tumors [2]. In *Xenopus laevis* oocytes, microinjection of oncogenic (containing Val in place of Gly 12), but not wild-type, p21 induces oocyte maturation [3]. Insulin induces oocyte maturation and requires activation of normal cellular *ras*-p21 [4].

Several agents that strongly block Val 12-p21-induced oocyte maturation have virtually no effect on insulin-induced maturation [5]. Among these agents are specific peptides, identified from molecular modeling studies, that correspond to effector domains from both *ras*-p21 itself, such as the 35–47, 96–110 and 115–126 sequences [5] and from some of its target proteins such as the *ras*-binding domain of *raf* (residues 97–110) [6, 7, 8] and the SOS guanine nucleotide exchange protein (residues 994–1004) [9, 10]. These peptide domains were identified as those that change conformation in response

to the presence of single oncogenic amino acid substitutions at positions 12 or 61 or multiple substitutions at positions 10, 12 and 59 when the computed average structures for these proteins either alone or in complex with target proteins were superimposed on that for the wild-type protein.

Our finding that these peptides (in addition to other agents) block oncogenic *ras*-p21 selectively suggested to us that the oncogenic protein induces mitogenesis by pathways that may overlap with but are also distinct from pathways utilized by the wild-type protein. In studies designed to identify pathway differences, we found that oncogenic but not insulin-activated wild-type *ras*-p21 interacts with the transcriptional activating protein, *jun* and its kinase, *jun* kinase (JNK) [11, 12], and requires the presence of protein kinase C (PKC) [13]. In these studies, we found that the peptide whose sequence corresponds to p21 residues 96–110, called PNC-2, blocks the interaction of Val 12-p21 with JNK [11, 12] in a dose-response curve that superimposes on that for its inhibition of Val 12-p21-induced oocyte maturation [5]. This suggested to us that one important site of action of PNC-2 is the interaction of Val 12-p21 with JNK [5].

Additionally, the peptide whose sequence corresponds to p21 residues 35–47, called PNC-7, encompasses a domain of the protein implicated in its interaction with multiple targets including *raf* p74 protein, GTPase-activating protein (GAP) and the guanine nucleotide exchange protein, SOS (reviewed in reference 5). This peptide strongly inhibits c-*raf*-induced oocyte maturation but has no effect on oocyte maturation induced by an oncogenic mutant *raf* lacking the *ras* binding domain (RBD) in its amino terminal regulatory domain [14, 15]. These findings suggest that an important site of action of this peptide is *raf* by binding to its RBD. Thus, both PNC-2 and PNC-7 appear to act on different steps on the oncogenic *ras*-p21 signal transduction pathway.

Selective inhibition by PNC-2 and PNC-7 of oncogenic *ras*-p21-induced oocyte maturation suggested to us that these peptides might be able to block the growth of *ras*-transformed mammalian cancer cells but not that of their untransformed counterpart cells. We have developed a new pancreatic acinar cell line, BMRPA1, that forms stable contact-inhibited monolayers and behaves phenotypically like acinar cells with respect to such functions as response to secretagogues [16, 17]. We have transfected these cells with the k-*ras* oncogene (encoding Val 12-p21) and developed a stably transformed pancreatic carcinoma cell line, TUC-3, that displays the transformed phenotype and forms metastatic tumors in nude mice [16, 17]. A major motivation for selecting the pancreatic cell model was the finding that over 90% of pancreatic carcinomas express oncogenic Val 12-p21 protein [2, 18].

In this study, we treated both cell lines with PNC-2 and PNC-7 peptides attached to a penetratin sequence enabling cell membrane penetration [19, 20] and with plasmids encoding each peptide sequence in order to determine whether these peptides can selectively block the growth of *ras*-transformed pancreatic cancer cells.

## Materials and methods

### Peptides

Three peptides, attached to the penetratin leader sequence, KKWKMRRNQFWVKVQRG, designated as "leader", on their carboxyl terminal ends, were synthesized by solid-phase methods: the two *ras*-p21 peptides corresponding to p21 residues 35–47 (TIEDSYRKQVVID) and 96–110 (YREQIKRVKDSDVP), denoted as PNC-7 and PNC-2, respectively; and the negative control X13 sequence (from mammalian cytochrome P450) (MPFSTGKRIMLGE). With the penetratin sequence attached to their carboxyl terminal ends, these peptides are denoted PNC-7-leader, PNC-2-leader and X13-leader, respectively. All peptides were purified to >95% purity.

### Plasmids

Construction of the plasmids that express the Ha-*ras* Val 12-p21 peptide sequence 96–110 (PNC-2) and the control X13 peptide from mammalian cytochrome p450 has been described previously [21]. The nucleotide sequences for PNC-2 and X13 peptides are given in reference 21. The nucleotide sequences, including the 5′ sticky end, used to encode the PNC-7 peptide were 5′-T CGA GCC ACC ATG GGG ACC GAG GAT TCT TAC AGA AAA CAA GTG GTT ATA GAT TAA C and 3′-CGG TGG TAC CCC TGG TAT CTC CTA AGA ATG TCT TTT GTT CAC CAA TAT CTA ATT GGG CC. Briefly, all of the oligonucleotides (plus and minus strands) encoding each sequence (PNC-2, PNC-7 and X13) and including a Not1(5′) and Kpn1(3′) restriction site were synthesized by solid-phase methods; sequential degradation of each oligonucleotide confirmed its sequence. These oligonucleotides were then incorporated into the pOPRSVI/MCS vector from the Lac switch II isopropylthioglucose (IPTG)-inducible mammalian expression system from Stratagene (La Jolla, Calif.) by cutting this vector with Kpn1 and Not1 and then ligating the oligomers into the plasmid with T4 ligase overnight at 4°C. The vectors containing the cloned oligonucleotides were transfected into DH5α-competent cells (Gibco-BRL, Grand Island, N.Y.) and spread on LBamp plates for overnight incubation. Colonies from each plate were selected and grown at 37°C in 5 ml LBamp liquid medium. DNA was prepared by the Miniprep procedure (Qiagen, Valencia, Calif.), cut with Kpn1/Not1, and run on 2% agarose/Tris-acetate-EDTA to estimate the size of the inserts. Clones with the correct size DNA inserts were regrown in 500 ml LBamp overnight at 37°C, and plasmids were then purified by the Qiagen Maxiprep method. An aliquot of each positive DNA was sequenced using T3 or T7 primers.

We note that, in our former paper describing these plasmids, an error occurred in the 5′ nucleotide sequence encoding PNC-2. This sequence should have read:

— *Upper*: 5′-CGCCGCCATGGG**C**TACAGGGAGCAGATC-AAGAGGGTGAAGGACAGCGACGACGTGCCCTA

In our original paper the highlighted C was inadvertently omitted.

### Cells

As described in several prior reports [16, 17, 20], we have developed two cell lines, one a normal contact-inhibited line of rat pancreatic acinar cells, called BMRPA1.430 (BMRPA1) cells, and the other a pancreatic acinar carcinoma obtained by transfection of BMRPA1 cells with a plasmid containing an activated human K-*ras* oncogene [single base mutation at codon 12, valine substitution for the

wild-type glycine in the ras protein (K-ras$^{val12}$); a kind gift from Dr. M. Perucho (CIBR, La Jolla, Calif.)] and a neomycin resistance gene. BMRPA1 cells have an epithelial cell phenotype, form acinar structures in culture, have no c-ki-*ras* nor p53 mutations, are unable to grow in anchorage-independent conditions and do not form tumors in Nu/Nu mice [17]. In addition, they phenotypically maintain differentiated cell functions such as continued enzyme production and activation of zymogen secretion by secretagogue. On the other hand, *ras*-transformed BMRPA1 or TUC-3 cells, selected after transfection for their resistance to G418 and the overexpression of K-ras$^{val12}$, no longer display an epithelial cell phenotype and acinar cell functions. They grow significantly faster than BMRPA1 cells, have a transformed spindle cell phenotype and form colonies under anchorage-independent conditions in vitro and tumors in vivo in nude mice.

### Peptide incubation experiments

Approximately 300,000 cells (either BMRPA1 or TUC-3) were plated in each of six wells and were allowed to adhere overnight. In one set of experiments, the initial medium consisted of DMEM with 10% fetal bovine serum that contained no peptide. In another set of experiments, the initial medium contained peptide. In the first set, medium containing peptide was added after 24 h; in both sets, after the first 24 h, the medium was changed every 24 h and always contained peptide at a particular concentration. Cells were observed daily for 3 weeks for changes in morphology and growth characteristics. Peptides were present at concentrations of 1, 10, 50, 100 and 200 μg/ml.

### Transfection experiments

Approximately 300,000 TUC-3 cells were plated in a six-well dish and were allowed to adhere overnight. To three wells, 5.5 μg of either PNC-2 or PNC-7 plasmid was added and, to the other three wells, 5.6 μg of X13 plasmid was added. To each of these wells, Superfect transfection agent (Qiagen) was added, using the Qiagen protocol, to enhance transfection efficiencies. We found that a 1:2 ratio of plasmid DNA to Superfect reagent gave the highest transfection efficiencies when compared with 1:5 and 1:10 ratios. Treated cells were then plated in selective medium containing 100 μg/ml G418 and 200 μg/ml ampicillin together with 1 m*M* IPTG. The cells were washed and the medium changed every 24 h. Viable cells were observed for morphology and growth characteristics over a 2-week period.

### Explantation of cells into nude mice

To evaluate cells that appeared to be morphologically revertant to the normal phenotype, approximately $5\times10^6$ morphologically revertant TUC-3 cells treated for 2 weeks with 100 μg/ml PNC-2 were injected subcutaneously into the posterior cervical fatpad of each of five nu/nu mice. Similarly, $5\times10^6$ untreated TUC-3 cells were explanted into another five nu/nu mice. Daily observations over were made over 120 days on both sets of mice to determine if tumor nodules appeared at the site of injection.

## Results

### Effects of peptides on TUC-3 and BMRPA1 cells

Figure 1A shows the morphology of untreated TUC-3 pancreatic carcinoma cells and Fig 1C that of their normal counterpart BMRPA1 pancreatic acinar cells. The former were not-contact-inhibited and did not form monolayers but were "heaped up" on one another with considerable pleomorphism between cells and indistinct cell boundaries. The latter formed contact-inhibited monolayers with well-defined cell boundaries. Incubation of the X13-leader control peptide with TUC-3 cells for 2 weeks had no effect on their transformed morphologies (Fig 1B). As expected, incubation of this control peptide with BMRPA1 cells had no effect (not shown). Incubation of BMRPA1 cells with PNC2-leader peptide likewise had no effect on the morphology of these cells (Fig. 1D).

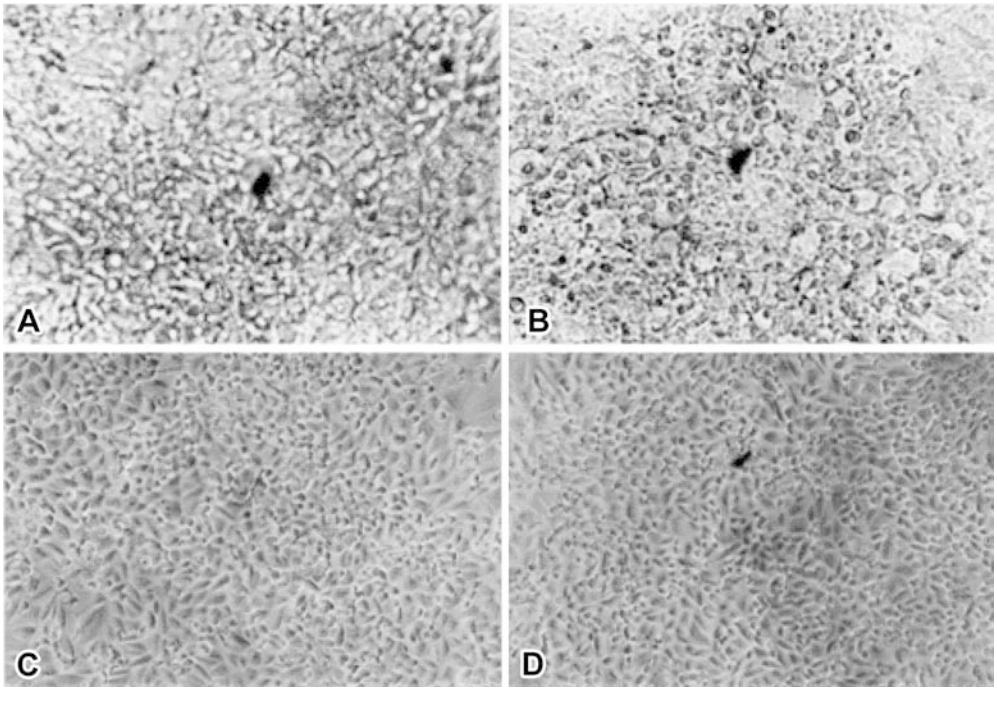


**Fig. 1A–D** Effects of different peptides on the growth of pancreatic acinar (BMRPA1) cells and *ras*-transformed pancreatic cancer (TUC-3) cells (**A** untreated TUC-3 cells, **B** TUC-3 cells treated with X13-leader peptide for 2 weeks, **C** untreated BMRPA1 cells at confluence, **D** BMRPA1 cells treated with PNC-2-leader peptide) showing no change in morphology or cell viability

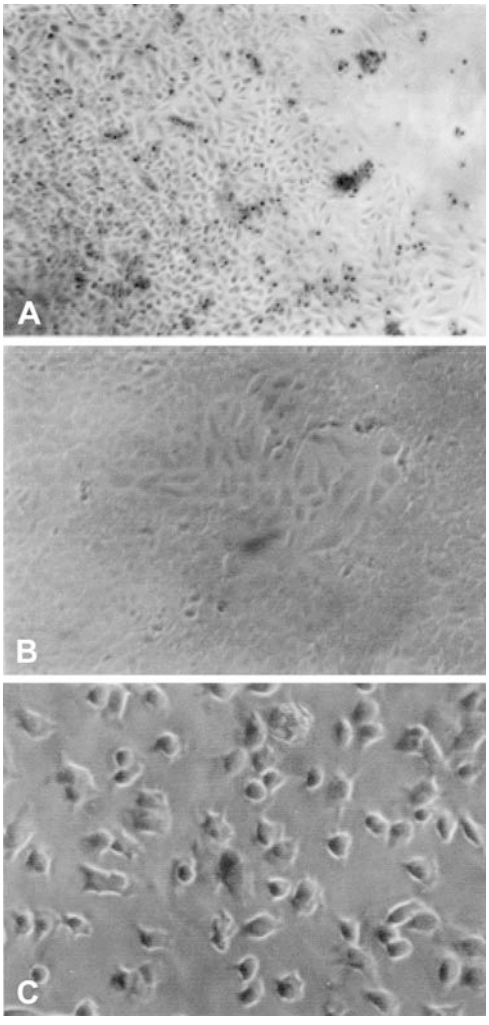


**Fig. 2A–C** Effects of PNC-2- and PNC-7-leader peptides on TUC-3 cells. **A** Effects of 100 μg/ml of PNC-2-leader on TUC-3 cells after 2 weeks of treatment. **B** Effects of 100 μg/ml of PNC-2-leader on TUC-3 cells after 1 day of treatment. In the center, a focus of morphologically revertant cells is apparent. **C** Effects of 100 μg/ml PNC-7-leader peptide on TUC-3 cells after 2 weeks of treatment

## Effects of PNC-2-leader and PNC-7-leader on TUC-3 cells

Treatment of TUC-3 cells with PNC-2-leader (100 μg/ml) for 1 week resulted in a change in cell morphology as shown in Fig. 2A. The cells appeared very similar to BMRPA1 cells (Fig. 1C); the cells grew into contact-inhibited monolayers and showed distinct cell boundaries. This effect was achieved at concentrations as low as 1 μg/ml. At this low concentration, complete phenotypic reversion was achieved after 2 weeks. After one day of treatment, foci of acinar cellular differentiation appeared; an example of a focus of revertant cells is shown in Fig. 2B.

Treatment of TUC-3 cells with PNC-7-leader peptide at concentrations of 100 and 200 μg/ml likewise resulted in phenotypic reversion of the cells, as shown in Fig. 2C for cells growing into confluence. In contrast to the results obtained with PNC-2-leader peptide, complete reversion after 2 weeks of incubation of TUC-3 cells with PNC-7-leader was achieved only at concentrations ≥100 μg/ml.

## Transfection of TUC-3 cells with inducible plasmids encoding PNC-2 and X13 peptides

Since both PNC-2- and PNC-7-leader peptides induced phenotypic reversion while X13-leader control peptide did not, we conclude that induction of reversion is specific to the two *ras*-p21 peptides and that the leader sequence, besides enabling membrane penetration, does not contribute to the induction of phenotypic reversion. To test the latter conclusion further, i.e. that PNC-2 and PNC-7 peptides alone, without the leader sequence, can induce phenotypic reversion, we prepared plasmids encoding these and the negative control X13 sequences and transfected them into TUC-3 cells. We have previously described the preparation of these plasmids which simultaneously confer G418 and ampicillin resistance under the lac promoter [21]. We co-microinjected these plasmids with Val 12-p21 protein into *Xenopus laevis* oocytes and found that oocytes injected with either PNC-2 or PNC-7 but not X13 plasmid, in the presence of IPTG, did not undergo maturation [21]. When we transfected each of these plasmids into TUC-3 cells growing in the selective medium, viable cells expressing X13 peptide continued to grow in the presence of IPTG and exhibited the transformed morphology shown in Fig. 1A.

On the other hand, during a period of 2 weeks post-transfection with PNC-2 plasmid, all viable TUC-3 cells became progressively differentiated as shown in Fig. 3A (after 1 week) and Fig. 3B (after 2 weeks). After 1 week, many cells adopted the untransformed phenotype (Fig. 3A, center and left) while some cells exhibited the transformed phenotype (Fig. 3A, right). At the end of 2 weeks, all cells exhibited the morphology shown in Fig. 3B. The cells had distinct cell boundaries and exhibit the same morphology as untransformed BMRPA1 cells in growth phase. These cells eventually grew into contact-inhibited monolayers with a morphology that was the same as shown in Fig. 1C.

TUC-3 cells transfected with PNC-7 plasmid exhibited the phenotype shown in Fig. 3C. These cells, which were enlarged with enlarged nuclei but had distinct cell boundaries, grew only sluggishly to confluence, and strongly resembled viable revertant cells that resulted from the treatment of TUC-3 cells with the PKC inhibitor, CGP 41 251 [16]. These cells fail to grow in soft agar [16].

## Morphologically revertant cells do not form tumors in nude mice

To test whether morphologically revertant cells were functionally revertant, $5\times10^6$ cells treated for 2 weeks

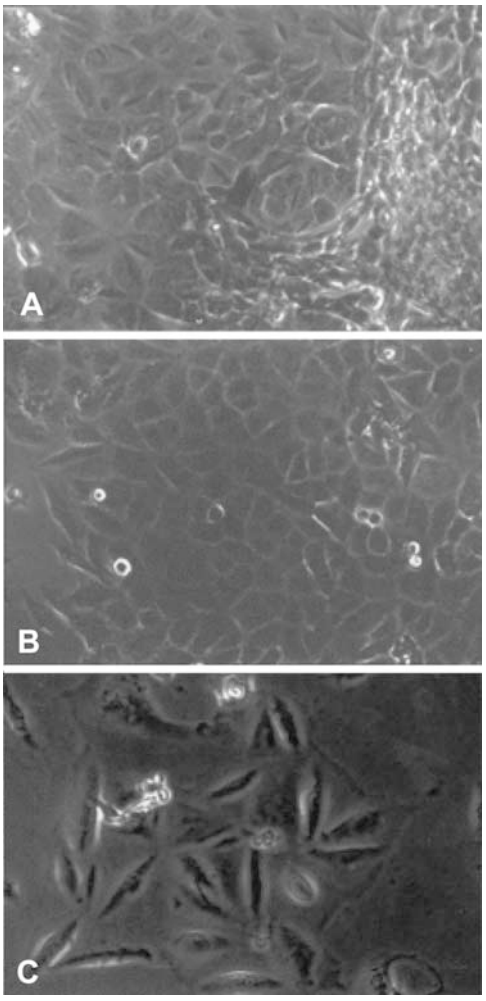


**Fig. 3A–C** Effects of transfection of lac-promoter-containing plasmid expressing either PNC-2 or PNC-7 into TUC-3 cells. **A** One week after plating transfected (with PNC-2-expressing plasmid) viable TUC-3 cells in selective medium, foci of reversion can be seen (*left* and *center*); *right* remaining transformed cells can be seen. **B** All transfected (with PNC-2-expressing plasmid) TUC-3 cells revert 2 weeks after transfection and selection of viable cells. **C** TUC-3 cells transfected with PNC-7-expressing plasmid, 2 weeks after transfection, showing cell and nuclear enlargement. These cells grow sluggishly into stable monolayers

with 100 μg/ml PNC-2-leader peptide, a concentration that ensures rapid and complete phenotypic reversion, were explanted subcutaneously into each of five nude mice, while the same number of untreated TUC-3 cells were concomitantly similarly explanted. Morphologically revertant cells failed to form tumors up to 2 months after reversion while untreated cells formed tumors rapidly (within 1 week) (Table 1). At 3 weeks, all of the nude mice injected with untreated TUC-3 cells were found to have large primary nodules and multiple other nodules and metastatic cancer, with ascites. Similar results (not shown) to those obtained with PNC-2-leader peptide-treated TUC-3 cells were obtained for morphologically revertant cells resulting from TUC-3 cells treated with PNC-7-leader peptide.

**Table 1** Growth of TUC-3 cells and morphologically reverted TUC-3 cells treated with PNC-2 peptide explanted into nude mice. TUC-3 cells ($5\times10^6$) were injected into the posterior cervical fat pad of each of five nude mice, and the same number of TUC-3 cells treated for 2 weeks with PNC-2-leader peptide were injected into the posterior cervical fat pad of another five nude mice

| Time (days) | Tumor nodule size (mm)[a] | |
|---|---|---|
| | TUC-3 cells | PNC-2-treated TUC-3 cells |
| 0 | 0.0 | 0.0 |
| 7 | $4.8 \pm 1.8$ | 0.0 |
| 14 | $11.7 \pm 2.3$ | 0.0 |
| 21 | $14.8 \pm 3.6$[b] | 0.0 |
| 28 | – | 0.0 |
| 42 | – | 0.0 |
| 56 | – | 0.0 |

[a] Expressed as the means ± SD for the five mice in each group
[b] Multiple nodules and tumor metastasis with ascites occurred in all five mice at this time. Further observations were therefore discontinued

## Discussion

Both PNC-2 and PNC-7 peptides block mitogenic signaling by oncogenic *ras*-p21 in oocytes but have little effect on signaling by insulin-activated wild-type cellular p21 [5]. This finding suggested to us that growth of mammalian cells transformed by oncogenic *ras*-p21 could be selectively blocked by these peptides without affecting normal growth processes.

### Specificity of PNC-2 and PNC-7 peptides

We found that both peptides induced 100% phenotypic reversion of *ras*-transformed pancreatic (TUC-3) cancer cells and had no apparent effects on the growth of the normal counterpart BMRPA1 cell line. This effect was specific since neither the X13-leader control peptide nor the plasmid encoding it had any effect on TUC-3 cell proliferation. That the PNC-2 and PNC-7 sequences and not the leader sequence are responsible for this effect is supported by the absence of any effect on TUC-3 cells of the X13-leader peptide and by the finding that the plasmids encoding PNC-2 and PNC-7 without the leader sequence induced the same observed phenotypic reversion.

### PNC-2 and PNC-7 peptides induce lasting effects

A surprising finding was that the phenotypic reversion induced by both peptides occurred over a prolonged period of time (120 days), as revealed by the absence of any tumor growth of these cells when explanted into nude mice. Since the half-lives of these peptides was expected to be much shorter than 2 months, their effects are not likely to have been caused by their continuing presence. Significantly, the prolonged reversion effect appears to be independent of the site of action of these

peptides since PNC-2 blocks oncogenic *ras*-p21-JNK interactions [5, 11, 12] while PNC-7 blocks oncogenic *ras*-p21-*raf* interactions [14, 15].

It is possible that both peptides activate rapid expression of other proteins that interfere with oncogenic *ras*-induced cell proliferation. This type of effect has been observed in human pancreatic carcinoma cells induced to revert by the agent azatyrosine that is known to induce expression of the *ras* recision gene (*rrg*) [22, 23] and which also selectively blocks oncogenic *ras*-p21-induced oocyte maturation [13]. Another possibility is that each peptide, by blocking signal transduction unique to the oncogenic *ras*-p21-induced pathway, allows other inhibitory processes continuously to deactivate critical elements in this pathway.

## Contrast of the effects of PNC-2 and PNC-7 with a PKC inhibitor that selectively blocks oncogenic *ras*-p21

The activity of both PNC-2 and PNC-7 peptides contrasts with that of another oncogenic-*ras*-p21-specific inhibitor, the staurosporine derivative, CGP 41 251, that selectively inhibits PKC [24]. This agent blocks oncogenic *ras*-p21-induced oocyte maturation but has much less effect on insulin-activated wild-type *ras*-p21-induced maturation [13]. In contrast to the results with PNC-2- and PNC-7-leader peptides, this agent induces both necrosis and phenotypic reversion of TUC-3 cells [16] and is cytotoxic to BMRPA1 cells, although surviving cells grow rapidly into stable monolayers [16]. Cytotoxicity of CGP 41 251 may be due to its blocking critical PKC-dependent cell processes that may not be involved in cell proliferation.

In prior studies, we have found that PKC and JNK require each other's presence on the oncogenic *ras*-p21 signal transduction pathway [25]. We have further found that PNC-2 synergizes with CGP 41 251 in TUC-3 cells in that it significantly lowers its $IC_{50}$ for induction of cytotoxicity to a level that is not toxic to BMRPA1 cells [16]. This finding suggests the possibility that PNC-2, which blocks *ras*-p21-induced activation of JNK [5], inhibits the mutual PKC-JNK activation cycle, thereby removing an important activation process, resulting in facilitation of inhibition by CGP 41 251.

Evidently PNC-2 and PNC-7 exert a more selective effect that is specific to the oncogenic *ras*-p21 pathway, hence the lack of cytotoxicity of these peptides. This finding suggests that these peptides may be useful in the treatment of *ras*-induced human tumors. Since a high proportion of human pancreatic cancers contain the *ras* oncogene, these peptides may be effective in the treatment of this disease.

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