

In vitro inhibition of angiogenesis by prostasomes

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Prostasomes are biologically active organelles that are secreted by human prostate epithelial cells, and it is believed that they have a role in prostatic disease. We studied the effect of prostasomes on the human umbilical vein endothelial cell (HUVEC)/Matrigel model of angiogenesis, and the association of labelled prostasomes with HUVECs. The growth inhibitory effect of prostasomes on HUVECs was assayed by spectrophotometric measurement of residual biomass. Preparations of HUVECs on a Matrigel base were exposed to prostasomes, and the development of capillary-like networks was quantified. Prostasomes were labelled with PKH-26, and cultured with HUVECs. Prostasomes were not shown to have a significant effect on HUVEC survival. Angiogenesis assays showed inhibition. The PKH-26-labelled particles were shown to have adhered to the HUVECs. This study adds the inhibition of an *in vitro* correlate of angiogenesis to the known actions of prostasomes.

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Introduction

Prostasomes are biologically active organelles that are secreted by prostatic epithelial cells. They appear freely in semen, and have been shown to have an important role in fertility, with actions on sperm motility,¹ the acrosome reaction,² and immunomodulation.³ Prostasomes have also been shown to have an antioxidant capability, albeit indirectly exerted.⁴ Proteomic analysis has shown prostasomes to contain upwards of 130 proteins, including tissue factor, complement inhibitors, and dipeptidyl peptidase IV which has shown to be involved in activation and deactivation of growth factors and cytokines.^{5–7} The membrane characteristics of prostasomes are also unusual, being particularly rich in cholesterol and sphingomyelin.⁸

Prostasome-like particles have also been shown to be produced by prostate cancer cells, both as cell lines in culture,⁹ and by *in situ* prostate cancer, including metastatic deposits.¹⁰ Antibodies to prostasomes have been detected in the serum of patients with prostate

cancer,¹¹ suggesting that prostasomes are able to get from the prostatic ducts into prostate tissue in this disease, though recent work in our unit has failed to reproduce these results.

Owing to the wide range of bioactive proteins associated with prostasomes, it has been postulated that they may have an effect on prostate disease. The recent review by Ronquist and Nilsson¹² has speculated on promalignant properties through cell transformation, immunosuppression, proliferation, facilitation of local invasion, and angiogenesis. Despite this, relatively little work has been published on the area. Carlsson *et al*¹³ have shown that prostasomes exert a growth-inhibitory effect on a range of prostate cancer cell lines in culture: our own work has confirmed this.

To explore another aspect of the possible effect of prostasomes on development of malignancy, we have investigated the effect of prostasomes on angiogenesis. We have used the model of human umbilical vein endothelial cells (HUVECs) on Matrigel (a solubilized basement membrane preparation), and developed a method of quantifying angiogenetic activity. We have also looked at the impact of prostasomes on HUVEC survival, and demonstrated the physical interaction of prostasomes and HUVECs through a fluorescent labelling technique using one of the family of the aliphatic, lipid-like PKH markers.

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Materials and methods

Cell culture

HUVECs were maintained in Medium 200 (Cascade Biologics), supplemented with L-glutamine–penicillin–streptomycin (Sigma, Poole, UK). The culture medium was changed twice a week, and the cells were trypsinized and subcultivated once a week. The human prostatic cancer cell line PC3 (originally derived from a bone marrow metastasis) was grown in RPMI 1640 medium (Sigma) enriched with 10% fetal calf serum and supplemented with L-glutamine–penicillin–streptomycin. These cells were trypsinized and subcultivated as they approached 100% confluence, typically twice a week.

Preparation of prostasomes

Prostasomes were isolated from pooled postvasectomy human semen, according to the protocol described by Ronquist and Brody.¹⁴ The semen was initially centrifuged at 10 000 g for 15 min to separate cells from the seminal plasma. The supernatant was then subjected to ultracentrifugation, at 100 000 g for 2 h at 4°C. The resulting pellet was resuspended in Ca²⁺/Mg²⁺-free Dulbecco's phosphate-buffered saline (Sigma). The suspension was further purified by Sephadex 200 (Sigma) gel chromatography in order to purify prostasomes from amorphous substance. The Sephadex column was equilibrated and eluted with PBS, at a flow rate of 0.5 ml/min. Fractions of 2.5 ml were collected. The presence of prostasomes in fractions was demonstrated by assay for amino-peptidase activity,¹⁵ and spectrophotometry at 280 nm.¹⁶ Prostate containing fractions were pooled, and the protein concentration measured.¹⁷ The suspension was subdivided into 1 ml aliquots and frozen at -20°C.

Preparation of PKH-26 labelled prostasomes

Semen-derived prostasomes were labelled with PKH-26, using the PKH-26 labelling kit (Sigma). A 1 ml aliquot of prostasome preparation had 2 ml of Diluent C from the labelling kit added to it. After 2 min, 1 ml of a 1:100 dilution of the PKH-26 label was added, followed 2 min later by 15 ml of serum-enriched RPMI 1640 medium. The prostasomes were recovered by ultracentrifugation at 100 000 g for 2 h at 4°C, and resuspension of the consequent pellet in PBS.

HUVEC survival assay

HUVECs were harvested and suspended in Medium 200. A volume of 100 µl of this suspension was placed in each of 25 wells of a flat-bottomed 96-well plate (Nunc, Fisher Scientific Loughborough, UK). After 24 h, 100 µl of prostasome preparations at a range of concentrations prepared in PBS was applied: the resultant prostasomal protein concentration in the wells were 0, 7.5, 47, and 375 µg/ml (Figure 1). After a further 24 h, the plates were visually inspected. A mesyure of 50 µl of a 1.25 mg/ml solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl

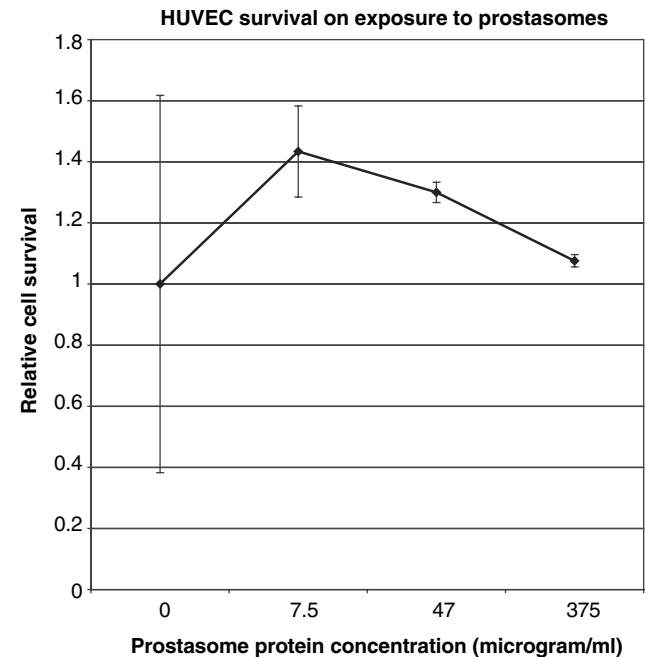


Figure 1 Plot of relative cell survival against prostasomal protein concentration. Error bars represent 95% confidence intervals. No significance of control compared to prostasome was observed.

tetrazolium bromide (MTT, supplied by Sigma) in RPMI was pipetted into each well. After 3 h, each well was aspirated to dryness, and then 150 µl of DMSO was added to each well. After agitation, absorbance at 550 nm (reference at 405 nm) was measured.

Angiogenesis assay

Frozen growth-factor depleted Matrigel (BD Biosciences, Oxford, UK) was warmed to 0°C, and a quantity sufficient to cover the base was carefully pipetted into the wells of a 96-well plate. After 1 h at 37°C, 50 µl of a suspension in Medium 200 of HUVECs at a concentration of 120 000 cells/ml was placed in each well. To this was added 50 µl of prostasome preparations at a range of concentrations prepared in PBS: the resultant prostasomal protein concentration in the wells were 0, 3, 15, 75, and 375 µg/ml. After 24 h, 50 µl of a 1.25 mg/ml solution of MTT in Medium 200 was added to each well to enhance visibility of the HUVECs. When uptake of the MTT had been allowed for 3 h, digital images of each well were taken using a Nikon digital camera through an invert microscope: the same zoom position was used for each image, although the focus of the microscope was adjusted to take account of the varying depths of matrigel. Each image was opened with Microsoft Paint (see Figure 2). A line was traced over all pseudotubules visualised, and from this the total pseudotubule length and network branches could be quantified (see Figure 3).

PKH-26 labelled prostasome binding

Portions of 1 ml of a suspension in Medium 200 of HUVECs at a concentration of 90 000 cells/ml were

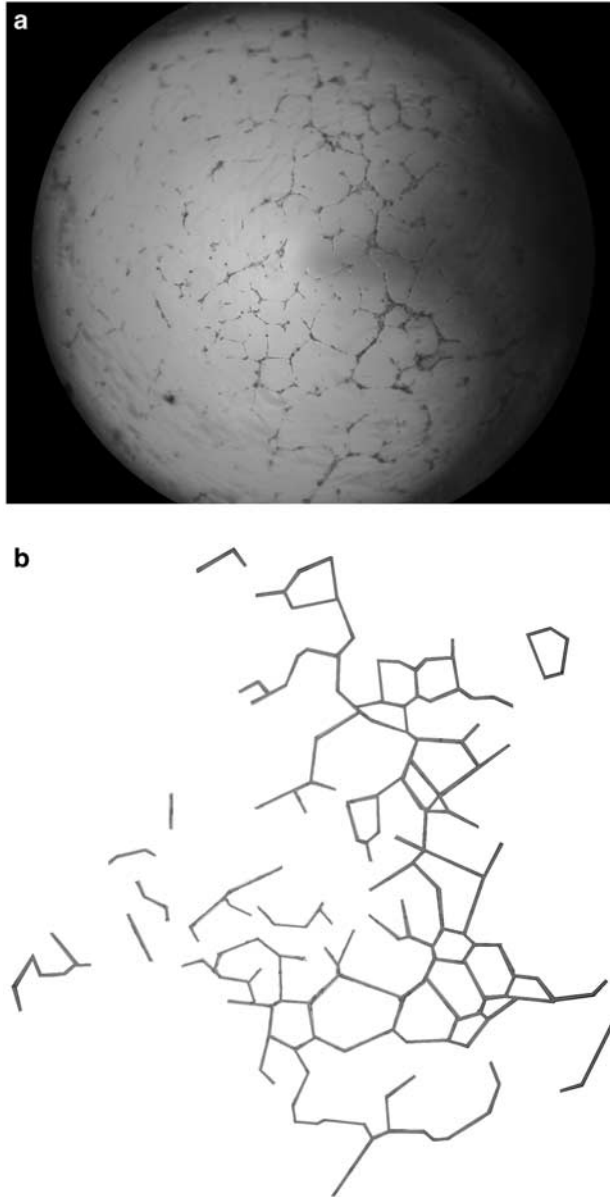


Figure 2 Analysis of angiogenesis-type activity. (a) Typical appearance of HUVECs on Matrigel after 24 h. (b) Trace of pseudotubule formation, which allows the quantification of pseudotubule length and number of network branches.

cultured in Petri dishes. After 24 h, 100 μ l of a suspension of prostasomes labelled with PKH-26 was added to each Petri dish. After being left overnight, the cells were examined using a laser scanning confocal microscope (LSCM) (Figure 4).

Results

HUVEC survival assay

The MTT assay is a quantitative colorimetric test to determine cellular proliferation. MTT is a yellow tetrazolium salt which is metabolised by mitochondrial succinic dehydrogenase activity of proliferating cells to yield a purple formazan reaction product. The quantity

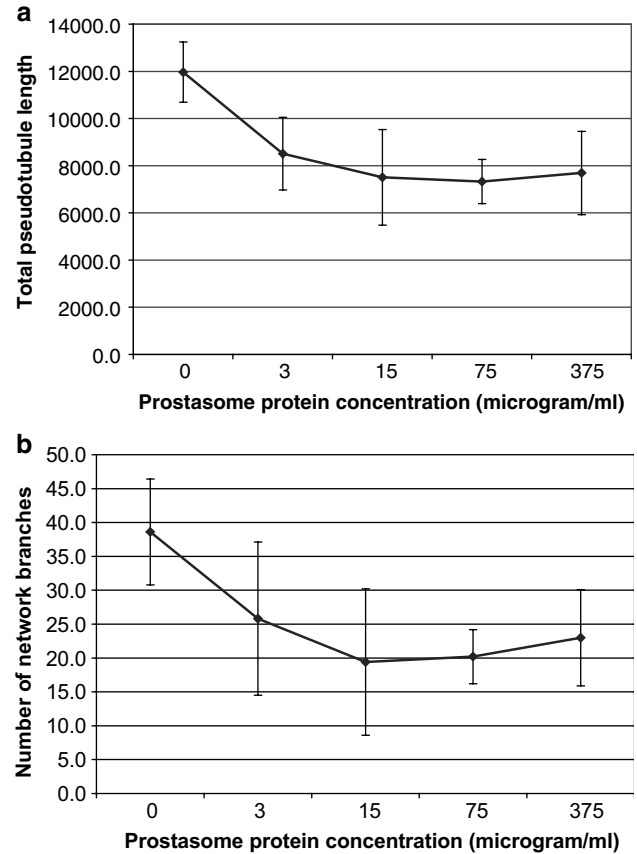


Figure 3 (a) Total length of pseudotubule formation against prostasomal protein (μ g/ml). (b) Network branches against prostasomal protein (μ g/ml). Error bars denote 95% confidence limits.

of this coloured reaction product can be assayed by measuring light absorbance at 550 nm.

No significant effect (as tested by the unpaired *t*-test) on HUVEC survival by exposure to prostasomes was demonstrated, with *P*-values greater than 0.35 for all prostasomal protein concentration compared to the control. This suggested that any effect on angiogenesis activity was not simply due to toxicity.

Angiogenesis assay

Measurement of total pseudotubule length showed significant (by the unpaired *t*-test) reduction at all concentrations of prostasome compared with the control with *P*-values all less than 0.007. Measurement of network branches also showed significant difference (*P* < 0.03) to the control for prostasomal protein concentrations of 15 μ g/ml: significance was not shown at 3 μ g/ml (*P* = 0.089). No significant differences were demonstrated between the different concentrations of prostasome for either pseudotubule length or network branches.

PKH-26 labelled prostasome binding

HUVECs were shown to fluoresce following exposure to PKH-26-labelled prostasomes, with no apparent differ-

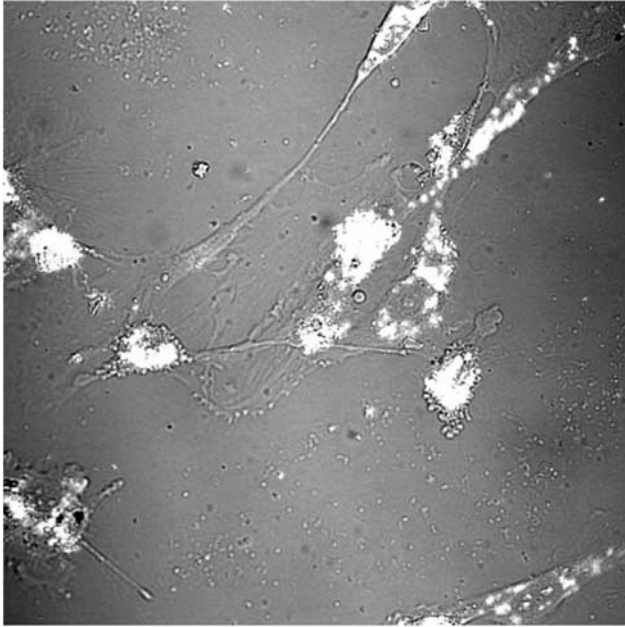


Figure 4 HUVECs after exposure to PKH-26-labelled prostasomes.

ence in appearances between prostasomes derived from semen or PC3 cell culture. It appeared that the fluorescent label was distributed in discrete particles over the cell, suggesting adherence rather than fusion, as described with other cell types.¹⁸

Discussion

The main finding of this current experiment was the unambiguous inhibition of the Matrigel/HUVEC correlate of *in vitro* angiogenesis by prostasomes, which does not appear to be simply due to inhibition of cell proliferation.

Our experiments with PKH-26-labelled prostasomes show that prostasomal membranes interact with those of HUVECs, in a similar manner to that previously described by others with spermatozoa and leucocytes. It has been demonstrated that prostasomes fuse with spermatozoa resulting in the loss of identifiable discrete prostasomal particles,¹⁸ resulting in the transfer of enzyme activity,^{19,20} altered membrane fluidity,²¹ and changes in the acrosome reaction.² This work has shown that these interactions are favoured at slightly acidic conditions: it occurs optimally at pH 5.0–5.5, and is abolished by pH 8.0. This compares with the reported average pH of semen of 7.6,²² although the first part of the ejaculate, containing the prostatic secretions and spermatozoa, is more acidic. Some interaction is observable at neutral conditions.

Arienti *et al*¹⁸ have also shown that prostasomes interact with leucocytes. Their fluorescence studies demonstrated that prostasomes adhered to, rather than fully integrated with, the leucocytes. Our labelling study suggests that this is what happens with HUVECs (and some of our other work suggests the same for prostate cancer cell types). The interaction with leucocytes was measured at pH 5.0 and 8.0 only, and was found not to occur at pH 8.0. Another study examined the functional

effect of prostasomes on neutrophils,²³ and found that prostasomes reduce NADPH oxidase activity in PMNLs, with the experiment carried out at pH 7.4. The authors postulate that this change in activity may be due to lipid transfer from the prostasomal membrane, resulting in interference vesicle trafficking and membrane-modifications following PMNL stimulation.

Our fluorescent labelling studies have demonstrated the interaction of prostasomes with HUVECs at a physiological PH, probably by adherence rather than fusion. As mentioned, other work we have done similarly suggests interaction with PC3 and PNT2 prostate cell lines. This interaction has been shown to allow trafficking of lipid between prostasome and cell, with effects on enzyme activity. It has yet to be demonstrated whether membrane-associated proteins are also transferable.

Extracellular membrane vesicles from tumour cells (EMVTCs) are particles shed from the surface of tumour cells. They sound very similar to prostasomes: they are rich in surface antigens and proteases, and have membranes containing increased amounts of cholesterol and sphingomyelin. Work with EMVTCs from the HT1080 fibrosarcoma cell line has shown promotion of angiogenesis (using an endothelial cell/matrix and chick chorioallantoic membrane models by a mechanism in which sphingomyelin is the major active component.²⁴ Preparations of EMVTCs, which were heat treated to degrade the protein content, were also shown to promote angiogenic activity, suggesting that protein exchange or activity is not a significant contributor to any effect. The paper describes the preparation of EMVTCs from the conditioned medium of the prostate cancer cell line DU145 in culture is described, and it is claimed that the EMVTCs derived from DU145 cells promote angiogenic activity in a similar manner to those derived from, on which the results are given, although interestingly no data derived from DU145 EMVTCs are given.

Our demonstration of a reduction in angiogenic activity contrasts with the previously suggested theory that prostasomes should promote angiogenesis.¹¹ The mechanism suggested by Ronquist and Nilsson in their review is firstly by the action of prostasomal-membrane bound angiotensin-1-converting enzyme (ACE), which catalyses the conversion of angiotensin I to the neovascularisation-promoting angiotensin II. Secondly, prostasomal tissue factor was thought to lead to the activation of thrombin (via the formation of a complex with activated factor VII, generating active factor X), which then stimulates the production of vascular endothelial growth factor.

It is possible that our *in vitro* model does not adequately reflect the *in vivo* situation, with some pathways not able to function due to a lack of intervening substrates or cooperating cells. In a cancer, only those prostasomes that pass back into tissue due to disrupted architecture will influence pathogenesis or disease progression. However, this result, taken with the finding of inhibition of prostate cancer cell lines in culture, does at least temper the view that prostasomes have only protumour properties. It is known that prostasomes have a range of functions, and that their prime function seems to be promotion of sperm survival and function. There is no reason to suppose that prostasomes need necessarily exert all their influences

on tumour development in the same direction: it is conceivable that prostasomes inhibit tumour cell growth and angiogenesis, while simultaneously promoting malignant transformation, immunosuppression, and local invasion. There is also the possibility that prostasomes secreted by malignant prostate cells have different actions to those from benign cells: Carlsson *et al*¹⁰ demonstrated similar but non-identical banding patterns on electrophoresis of prostasomal protein derived from benign and malignant sources. The relationship of prostasomes to EMVTCs seems to have been previously not remarked upon, and would bear further investigation also.

Further work needs to be done on all of these aspects of tumour development, ideally in systems better able to replicate the clinical situation.

References

- Arienti G *et al*. The motility of human spermatozoa as influenced by prostasomes at various pH levels. *Biol Cell* 1999; **91**: 51–54.
- Palmerini CA *et al*. Fusion of prostasomes to human spermatozoa stimulates the acrosome reaction. *Fertil Steril* 2003; **80**: 1181–1184.
- Kelly RW *et al*. Extracellular organelles (prostasomes) are immunosuppressive components of human semen. *Clin Exp Immunol* 1991; **86**: 550–556.
- Saez F, Motta C, Boucher D, Grizard G. Antioxidant capacity of prostasomes in human semen. *Mol Hum Reprod* 1998; **4**: 667–672.
- Utleg AG *et al*. Proteomic analysis of human prostasomes. *Prostate* 2003; **56**: 150–161.
- Fernandez JA, Heeb MJ, Radtke KP, Griffin JH. Potent blood coagulant activity of human semen due to prostasome-bound tissue factor. *Biol Reprod* 1997; **56**: 757–763.
- Schrimpf SP *et al*. Identification of dipeptidyl peptidase IV as the antigen of a monoclonal anti-prostasome antibody. *Prostate* 1999; **38**: 35–39.
- Arienti G *et al*. Fatty acid pattern of human prostasome lipid. *Arch Biochem Biophys* 1998; **358**: 391–395.
- Nilsson BO *et al*. Expression of prostasome-like granules by the prostate cancer cell lines PC3, Du145 and LnCaP grown in monolayer. *Ups J Med Sci* 1999; **104**: 199–206.
- Carlsson L *et al*. Characteristics of human prostasomes isolated from three different sources. *Prostate* 2003; **54**: 322–330.
- Nilsson BO, Carlsson L, Larsson A, Ronquist G. Autoantibodies to prostasomes as new markers for prostate cancer. *Ups J Med Sci* 2001; **106**: 43–49.
- Ronquist G, Nilsson BO. The Janus-faced nature of prostasomes: their pluripotency favours the normal reproductive process and malignant prostate growth. *Prostate Cancer Prostatic Dis* 2004; **7**: 21–31.
- Carlsson L *et al*. Growth-inhibitory effect of prostasomes on prostatic cancer cell lines in culture. *Eur Urol* 2000; **38**: 468–474.
- Ronquist G, Brody I. The prostasome: its structure and function in man. *Biochem Biophys Acta* 1985; **822**: 203–218.
- Laurell CB, Weiber H, Ohlsson K, Ranevic G. A zinc-dependent peptidase in prostatic organelles present in seminal plasma. *Clin Chem Acta* 1982; **126**: 161–169.
- Olsson I, Ronquist G. Nucleic acid association to human prostasomes. *Arch Androl* 1990; **24**: 1–10.
- Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–254.
- Arienti G *et al*. Interactions between prostasomes and leukocytes. *Biochem Biophys Acta* 1998; **1425**: 36–40.
- Arienti G *et al*. Transfer of CD26/dipeptidyl peptidase (E.C. 3.5.4.4.) from prostasomes to sperm. *FEBS Letters* 1997; **410**: 343–346.
- Arienti G *et al*. Transfer of aminopeptidase activity from prostasomes to sperm. *Biochem Biophys Acta* 1997; **1336**: 269–274.
- Carlini E *et al*. Fusion of sperm with prostasomes: effects on membrane fluidity. *Arch Biochem Biophys* 1997; **343**: 6–12.
- Raboch J, Skakov J. The pH of human ejaculates. *Fertil Steril* 1965; **16**: 252–256.
- Saez F *et al*. Prostasomes inhibit the NADPH oxidase activity of human neutrophils. *Mol Hum Reprod* 2000; **6**: 883–891.
- Kim CW *et al*. Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. *Cancer Res* 2002; **62**: 6312–6317.