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Original Paper

Epidermal Growth Factor (EGF) Increases the *In Vitro* Invasion, Motility and Adhesion Interactions of the Primary Renal Carcinoma Cell Line, A704

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Metastasis is a multistep process that involves alterations in a tumour cell's invasion, motility and adhesive capabilities. This study examined the effect of EGF on the *in vitro* invasion, motility and adhesion of the primary renal adenocarcinoma cell line, A704. Stimulation of the tumour cells by EGF (40 ng/ml) for a period of 24 h increased the *in vitro* invasion ($P = 0.040$) and motility ($P = 0.039$). Cell adhesion was examined on fibronectin, laminin, collagen IV and a 1:1:1 mix of the three extracellular matrix components. After EGF (40 ng/ml) stimulation, adhesion was significantly decreased on fibronectin ($P = 0.022$) and collagen type IV ($P = 0.026$), but increased on the 1:1:1 mix of extracellular matrix components ($P = 0.022$). The 92 kDa matrix metalloproteinase (MMP-9) present in the cell-conditioned medium was also increased after a 24 h stimulation with EGF (40 ng/ml) when measured. Hence, EGF can modulate the *in vitro* invasion, motility, adhesiveness and matrix metalloproteinase production in the A704 cell line, and subsequently may have a role in the metastatic potential of some renal carcinomas.

Key words: renal carcinoma, metastasis, EGF, invasion, motility, adhesion, metalloproteinase

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INTRODUCTION

METASTASIS, THE major cause of death and morbidity in cancer patients, is a complex multistep process in which the invasive potential, adhesion and motility capabilities of a tumour cell play a major role [1, 2]. These aspects of tumour cell behaviour allow the cell to move from its primary site of growth by the degradation of surrounding extracellular matrix and basement membranes, and to adhere and invade at secondary sites. Factors that induce alterations in these parameters may have a significant function in the metastatic process at some level.

One group of molecules known to be involved in the regulation of many cellular processes are the cytokines/growth factors, of which epidermal growth factor (EGF) is a member [3]. EGF is a single-chain polypeptide (6 kDa) that has a wide spectrum of activities including mitogenic, angiogenic and chemotactic effects on normal and tumour cells [4]. Moreover, EGF has been shown to increase the experimental metastatic potential, motility and *in vitro* invasion of a number of tumour cells [4, 5]. Overexpression of its receptor, EGF-R, has been linked with a number of premalignant and malignant diseases, including a possible role in the progression of some tumours [4]. Gastric cancer patients whose tumours express high levels of EGF and EGF-R have a poorer prognosis when compared to patients expressing lower levels [6]. Therefore, EGF and its receptor may play an important role in the progression of some forms of tumours.

The ability of a tumour cell to degrade extracellular matrix components allowing invasion to occur is influenced by the release of proteolytic enzymes. While a variety of proteases are likely to contribute to the degradation of extracellular matrix components, the matrix metalloproteinases, especially the type IV collagenases, 92 kDa (MMP-9) and 72 kDa (MMP-2), play a major role in the degradation of

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basement membranes. These type IV collagenases have been shown to be important in the *in vivo* and *in vitro* invasion of tumour cells [7, 8]. Moreover, their expression has been shown to be modulated by cytokine/growth factors [4, 8, 9].

In this study we examine the effect of exogenous EGF on the invasive potential, motility, matrix metalloproteinase production and adhesive capabilities of the primary renal adenocarcinoma cell line A704.

MATERIALS AND METHODS

Cell lines

The primary renal adenocarcinoma cell line, A704 (HTB 45), was obtained from the American Type Culture Collection. The cells were maintained in RPMI 1640 medium (Gibco) supplemented (all final concentrations) with L-glutamine (2-mM; Gibco), penicillin (100 IU/ml; Gibco), streptomycin (100 μg/ml; Gibco) and 10% foetal calf serum (FCS; Gibco).

Epidermal growth factor (EGF)

EGF (Gibco, U.K.) was reconstituted to 1 μg/ml in sterile phosphate buffered saline, pH 7.5 (PBS). The stock solution was diluted in the appropriate cell growth medium to achieve the desired concentration.

In vitro *invasion assay*

The *in vitro* invasion assay was carried out as previously described [10, 11]. Briefly, an 8.0 μm polycarbonate filter membrane insert (Costar, U.K.) was coated with 15 μg (45 $\mu g/cm^2$) Matrigel (Collaborative Research, Becton Dickenson, U.K.). Confluent cells, which had been previously stimulated for a 24 h period with EGF (40 ng/ml), were harvested non-enzymatically by scraping, washed in serum-free RPMI 1640 medium with 0.1% BSA (bovine serum albumin), and added to the top of the chamber (3×10^5 cells/chamber). Conditioned MRC-5 fibroblast medium was used as a chemoattractant and was added to the bottom of the chamber. Chambers were incubated in a humidified incubator at 37°C in 5% CO_2 in air for 6 h. Cells remaining on the upper filter surface were mechanically removed by cotton swab and complete removal monitored by light microscopy. Cells that had traversed the Matrigel were fixed with 3% gluteraldehyde in PBS, stained with haematoxylin and counted under the microscope ($\times 200$). Invasion was calculated as the number of cells invaded/graticule field. Five/ten random graticule fields (973 μm^2) were counted per filter, the mean reading indicating the invasion potential as previously described [9]. Assays were performed in duplicate and repeated three times.

Motility assays

Polycarbonate filter membranes (8 μm) were precoated with 5 μg collagen type IV in a volume of 50 μl on the lower surface, and dried at room temperature. Cells (10^5 cells/filter) were seeded in the upper chamber, as described above, with EGF (40 ng/ml) and incubated for a period of 24 h at 37°C, 5% CO_2. After the period of incubation, the membrane filters were removed and treated as described for the invasion assays. The cell motility is expressed as the number of cells/graticule field with five/ten random graticule fields counted per filter. Assays were performed in triplicate.

Adhesion assays

The adhesion assay was performed as previously described [11]. Briefly, 96 well plastic plates (Costar, U.K.) were coated (10 μg/ml) with fibronectin, laminin, collagen IV (Sigma, U.K.), and a 1:1:1 mix of the aforementioned. Cells were stimulated for a period of 24 h with EGF (40 ng/ml) in RPMI 1640 medium supplemented with 10% FCS and then harvested by scraping. The cells were then washed in RPMI 1640 with 0.1% BSA, resuspended in this medium and plated at 10^5 cells/100 μl. After a 2 h incubation, non-adherent cells were removed by washing in PBS and the adherent cells were quantified by crystal violet staining. Cells were stained with 0.5% crystal violet (Sigma, U.K.) in 25% methanol for a period of 10 min. Excess stain was removed by gentle washing with tap water and the plates allowed to dry for 12–24 h. The crystal violet pigments were then solubilised by the addition of 0.1 M trisodium citrate in 50% ethanol for a period of 20 min. The optical density (OD) of the samples were read on a Titretek Multiscan MCC/340 plate reader using dual filters (540 nm absorbance, 405 reference). There is a linear relationship between the cell number and OD of the crystal violet stain [12] allowing quantification. Assays were carried out five times for each stimulation and each stimulation was done in triplicate.

Gelatin zymography

Gelatin zymography was performed as described by Seftor [13]. Cells were seeded in standard growth medium on 24 well plates and allowed to divide until confluence had been reached. Cells were then treated with fresh serum-free growth medium with or without EGF. Cells were incubated at 37°C, 5% CO_2 for 24 and 72 h. The conditioned media were then removed and concentrated by vacuum desiccation; samples were reconstituted in 75 μl of non-reducing buffer and incubated at 37°C for 1 h before being electrophoresed on non-reducing sodium dodecyl sulphate acrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed in 2.5% Triton X100 for 1 h and then washed thoroughly in distilled water. The gels were then incubated at 37°C in 50 mM Tris–HCl, pH 7.6 containing 10 mM calcium chloride, 0.15 mM sodium chloride, 1 μm zinc chloride and 0.02% sodium azide. After an incubation of 48 h, the gels were stained in 0.5% Coomassie brilliant blue R (BDH, U.K.) in acetic acid : isopropanol : water (1:3:6) and destained in ethanol (12%) and acetic acid (7%) overnight. Matrix metalloproteinase activity was visualised as bands of lysis on the gel and documented by photography.

Proliferation assay

The proliferation assay was carried out using the commercially available Cell Proliferation Kit (MTT) (Boehringer Mannheim Biochemica, U.K.). The method was according to the manufacturer's instructions.

Statistical analysis

The significance of differences between groups was calculated by the Student's two-tailed *t*-test.

RESULTS

Effect of EGF on in vitro *invasiveness*

Confluent A704 cells were stimulated with EGF (40 ng/ml) for a period of 24 h. The *in vitro* invasion potential of the cells was then measured as described. Stimulation with EGF (40 ng/ml) substantially increased the *in vitro* invasiveness of the A704 cells from 2.35 ± 0.64 (SEM) cells invaded/graticule field to 5.80 ± 1.32 (Figure 1a). Although the invasion potential of the cells over the 6 h period of study appears to be small, the effect of EGF represents a considerable increase in *in vitro* invasion of almost 1.5-fold, a significance of $P = 0.040$ when compared with the control samples.

Effect of EGF on motility

Analysis of A704 motility was investigated over a period of 24 h with the presence of EGF (40 ng/ml). The motility of the A704 cells was significantly increased ($P = 0.039$) from a mean value of 78.53 ± 4.10 cells/graticule field for the unstimulated cells to 95.53 ± 3.84 ($P = 0.039$) for cells stimulated with EGF (40 ng/ml) (Figure 1b). This represented an increase in motility of over 21% with EGF stimulation.

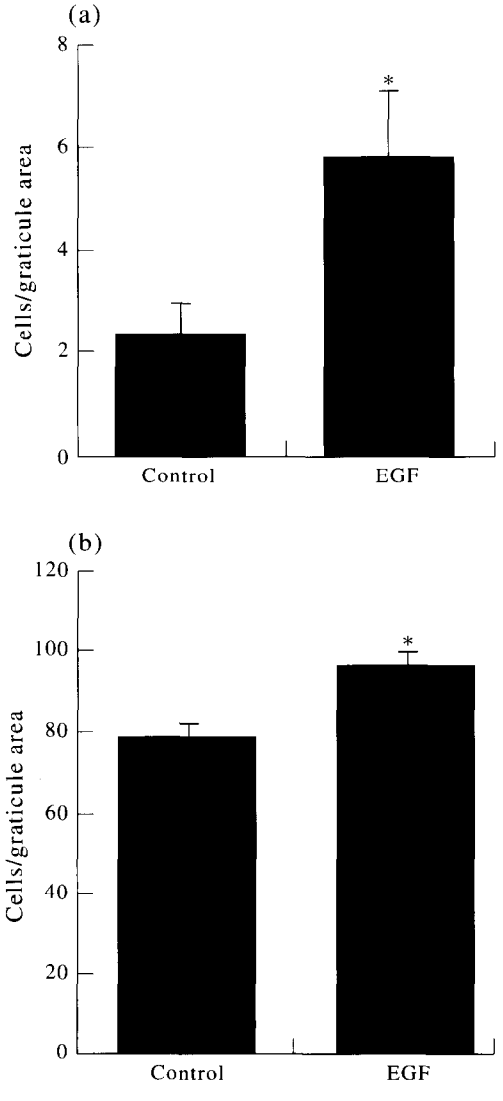

Figure 1. The effect of EGF (40 ng/ml) on (a) *in vitro* invasion and (b) motility after a 24 h exposure period. The data are expressed as cells invaded/graticule field (973 μm^2) and represent mean $\pm$ SEM ($n = 3$). The *in vitro* invasion and motility of the A704 cells was significantly increased upon EGF stimulation as compared with control cells (unstimulated) (*$P < 0.05$; Student's *t*-test).

Epidermal growth factor is known to increase the proliferation of many cell types [4]. To ensure that any significant increases in motility were not due to a cytokine/growth factor proliferative effect, the effect of EGF (40 ng/ml) was examined upon the proliferation of A704 cells over a period of 24 h. Cell proliferation was determined using the commercially available MTT assay (Boehringer Mannheim, U.K.).

No significant differences were found between the cell numbers of EGF-stimulated cells and control cells after a 24 h period as determined by statistical analysis (Table 1). Therefore, as the doubling time of the cell line is less than 24 h, it can be said that EGF does not have an effect on the proliferation of the cell line during the time period to produce any significant differences in the respective cell numbers. Thus, the observed increase in cell motility is not due to an increase in cell number in EGF-stimulated cells.

The effect of EGF upon adhesion

The effect of exogenous EGF on the adhesive capabilities of the A704 cell line was examined upon a variety of extracellular matrix substrates as a variety of interactions by one cell can occur on a variety of substrates. Cells were stimulated with EGF (40 ng/ml) for a 24 h period and their adhesion examined when plated on fibronectin, laminin, collagen IV and a 1:1:1 mix of the matrix components. The adhesion of the cells was significantly decreased on fibronectin and collagen type IV (Figure 2). On fibronectin, the decrease in adhesion was from 2.115 ± 0.073 (SEM) units of absorbance (equivalent to the number of adherent cells) to 1.869 ± 0.072 ($P = 0.022$), and for collagen type IV, the decrease was from 2.182 ± 0.123 to 1.823 ± 0.092 ($P = 0.026$). These alterations in the adhesive ability of A704 cells due to EGF stimulation represented almost a 12% decrease upon fibronectin and almost a 17% decrease on collagen type IV (Fig. 2). The effect of EGF on the adhesion of the A704 cells on the mix of matrix substrates (1:1:1 mix) had an opposite effect, significantly increasing the adhesion of the cells from 1.195 ± 0.045 to 1.414 ± 0.077 ($P = 0.022$) for the EGF-stimulated (Figure 2). This change in adhesion represented over an 18% increase between the control and EGF-treated cells.

Table 1. A704 proliferation in the presence of EGF

	Absorbance (620/690)	*P*-value†
Control	0.868 ± 0.053*	n/a
EGF (40 ng/ml)	0.921 ± 0.033	0.398‡

Cells were stimulated with/without EGF (40 ng/ml) for a period of 24 h and proliferation determined by the MTT assay as described in Materials and Methods.

* Proliferation is expressed as absorbance units $\pm$ SEM ($n = 16$). † *P*-value determined by the Student's *t*-test. ‡ No significant differences were observed after 24 h.

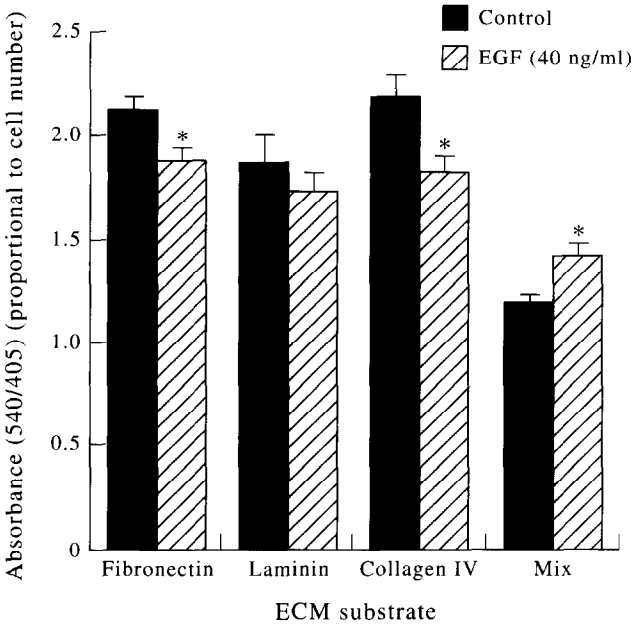

Figure 2. The effect of EGF (40 ng/ml) stimulation on the adhesion of A704 cells on fibronectin, laminin, collagen IV and a 1:1:1 mix. Data are expressed in absorbance units, which is directly related to adherent cell number [10] and represent mean ± SEM ($n = 3$). The adhesion of the A704 cells was significantly decreased on fibronectin and collagen IV, but significantly increased on the 1:1:1 mix after EGF stimulation ($*P < 0.05$; Student's t-test).

The effect of EGF on matrix metalloproteinase production

As described previously, EGF significantly increases the *in vitro* invasion of the A704 cells. A major contribution to the degradation of basement membranes in invasion is made by the family of matrix metalloproteinases of which 92 kDa (MMP-9) and 72 kDa (MMP-2) are members. The effect of EGF (40 ng/ml) on the production of these enzymes from A704 cells over a 24 h incubation period was examined. Control samples at 24 h showed that the 92 kDa matrix metalloproteinase was the most abundant with very little of the 72 kDa form being produced. When cells were stimulated with EGF (40 ng/ml) for a period of 24 h, the amount of 92 kDa metalloproteinase activity was greatly increased (Figure 3), but no effect upon the production of 72 kDa metalloproteinase was observed. As previously demonstrated, EGF does not significantly increase the proliferation of the cell line over a 24 h period; therefore, the increased levels of 92 kDa were not due to an increase in cell numbers in EGF-stimulated samples. The increases in 92 kDa metalloproteinase levels are consistent with the observation that EGF increases the *in vitro* invasion of the A704 cell line.

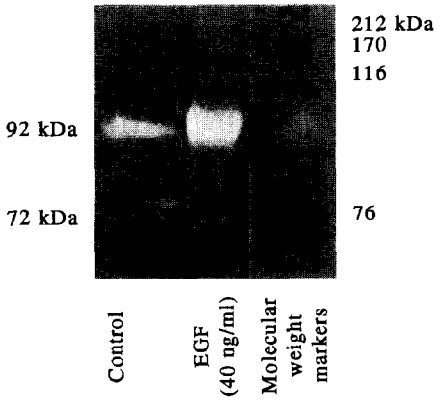

Figure 3. The effect of EGF (40 ng/ml) stimulation on the production of the 92 kDa and 72 kDa forms of metalloproteinases after a 24 h incubation period without and with EGF (40 ng/ml).

DISCUSSION

Metastasis is a complex process requiring the degradation of basement membranes and organ parenchymal matrix by the activation and secretion of proteases, enhanced motility, and changes in tumour cell adhesion [14]. A variety of agents have been shown to stimulate these aspects of tumour cell biology *in vitro*, and include cytokines/growth factors, tumour secreted factors, host derived scatter factors and components of the extracellular matrix [2].

EGF is a potent cytokine/growth factor that can modulate many aspects of normal and tumour cell behaviour. Present in the tumour micro-environment, EGF is postulated as having an important role in the progression and dissemination of some forms of tumour. The EGF receptor, which is overexpressed in some forms of tumour, may extenuate the sometimes detrimental effects of EGF and result in a poor prognosis amongst cancer patients [4, 6]. However, although it has been suggested that EGF may play a role in the spread of tumours, how this is achieved has not been fully delineated.

This study demonstrates that stimulation of a primary renal adenocarcinoma cell line, namely A704, with exogenous EGF (40 ng/ml) significantly effects the *in vitro* invasion, motility and adhesive ability of the cells. This triad of cellular traits plays a vital role in the invasion and metastatic potential of tumour cells. Moreover, we have shown that EGF increases the 92 kDa metalloproteinase, which parallels the increases in the *in vitro* invasion of the tumour cells. Therefore, we postulate that EGF has a role in modulating the invasive potential and metastasis of renal cells *in vivo*.

The specific change in the metalloproteinase production demonstrated in this study is an increase in the 92 kDa (MMP-9) production from the cells. In addition to the increase in latent 92 kDa metalloproteinases demonstrated here, EGF is known to increase the secretion of other proteases, such as plasminogen activator (PA) [15]. This protease leads to the conversion of plasminogen to plasmin, which is also involved in degrading matrix as well as the activation of latent type IV collagenases. Therefore, increases in plasminogen activator and plasmin by EGF increases the amount of active type IV metalloproteinases and matrix degradation. EGF has previously been reported to increase mRNA production of the matrix metalloproteinase stromelysin [16], which can also activate latent 92 kDa MMP leading to an increase in matrix degradation. In addition to the direct stimulatory effects on protease production, EGF may also act indirectly by the upregulation of cell adhesion molecules. Previous studies have shown that adhesion molecules, such as the β1 integrins, play an important role in the metastasis of some tumour types [17, 18]. The importance may not only be due to their role in cell adhesion and motility, but also in the modulation of protease secretion. It

has been demonstrated that, upon ligand binding to the β1 integrin of α2β1, protease secretion is induced [19]. Therefore, increases in α2β1 expression may not only lead to altered adhesion and motility, but also facilitate increases in protease secretion enabling invasion to occur. Fuji and colleagues demonstrated that EGF stimulation of human squamous carcinoma (HSC-1) cells can upregulate the expression of α2β1 [20]. Moreover, in recent studies of the A704 cell line, we have demonstrated similar increases in α2β1 expression upon EGF stimulation (data not shown). Thus, EGF may indirectly increase protease expression by increasing the expression of cell surface adhesion molecules that are involved in protease secretion. Whether the increase in the *in vitro* invasion of A704 cells is due to these mechanisms still has to be elucidated. However, it is likely that the increase in the production of 92 kDa MMP, as demonstrated in our study, and its later activation, may be modulated by EGF.

The results presented in this study confirm earlier findings by Otani and colleagues [5] in which increases in the *in vitro* invasion and overall type IV collagenolytic activity in two renal tumour cell lines were observed upon EGF stimulation. However, specific changes in metalloproteinase production in conjunction with invasion, motility and adhesion alterations were not investigated.

The increases in the *in vitro* invasion as a result of EGF stimulation were also accompanied by significant increases in the motility of the A704 cell line. These increases were possibly lowered by the fact that, although efforts were made to obtain single cell suspensions, cell aggregates were still present in the motility assay reducing overall cell motility. Previously, EGF has been shown to increase the motility of corneal epithelial cells [21], human keratinocytes [22], primary gliomas [23] and human squamous carcinoma cells [20]. The increases observed in the human keratinocytes and human squamous carcinoma cells were accompanied by increases in the β1 integrin, α2β1. Alterations have also been observed in the expression of β1 integrins in the A704 cell line upon EGF stimulation (data not shown), but the significance of these changes has not been determined.

The adhesive capabilities of tumour cells is an important aspect at several stages of the metastatic process. In this study, we have shown that EGF decreases the adhesive capabilities of the A704 cells to fibronectin and collagen IV, has no effect upon laminin but increases the adhesion of the cells upon a 1:1:1 mix of the matrix components. Although the effects are marginal, the decrease in adhesion towards fibronectin by EGF stimulation may lead to an increased motility and detachment at the primary tumour site. The increased adhesion towards the mix of matrix components may represent an increased adhesion towards the basement membrane facilitating increased invasion at secondary sites. The apparent opposite effects of the stimulated cells when plated on individual and mixed-matrix components appears confusing. However, one explanation may be that unique binding motifs are created due to the components being mixed, and adhesion receptors towards these are upregulated by EGF stimulation. That is, the α6β4 integrin, which has been shown to have binding properties towards the basement membrane, may be upregulated, enhancing adhesion towards the mix and not towards the individual components [24]. In addition, our analysis of the β1 integrin receptors reveals that upon EGF stimulation, decreases in α1β1, α3β1 and α4β1 and increases in α2β1, α5β1 and α6β1 expression are evident (data not shown). These alterations in integrin expression and their subsequent interaction may lead to an enhanced adhesion towards the mix rather than the individual components. Further analysis of their expression and interaction may reveal interesting relationships between different integrins leading to such results. The degradation of the matrix components by the release of matrix degrading proteases may also provide an explanation of the adhesion results. As previously demonstrated, EGF increases the expression of the 92 kDa matrix metalloproteinase, which has specificity towards type IV collagen. Therefore, the decrease in adhesion towards collagen IV may be due to an increased degradation of the extracellular matrix component during adhesion, an aspect which would occur less readily in the mix of matrix components.

In addition to the modulation of cell surface receptors involved primarily in cell–substratum interactions, EGF has also been shown to modulate those involved in cell–cell adhesion. Shiozaki and colleagues demonstrated that EGF can alter E-cadherin-mediated cell–cell adhesion resulting in an increased cell dissociation and *in vitro* invasion [25].

Effects of exogenous EGF can only be achieved by its interaction with the EGF receptor. The magnitude of effect, therefore, may be directly related to the amount of EGF receptor expression [4]. Müller and colleagues demonstrated that blockage of EGF receptor by specific antibody interaction suppressed spontaneous melanoma metastasis in SCID mice [26]. Recently, measurement of EGF receptor status by immunohistochemistry revealed a low expression of the receptor in the A704 cell line (data not shown). The low, yet significant, effects upon all motility and adhesion in the A704 cell line may be directly related to the low receptor expression. Amplification/overexpression of the EGF receptor in the cell line by further tumorigenic progression may lead to enhanced EGF effects.

We have demonstrated that EGF effects the *in vitro* invasion, motility and adhesion of the renal carcinoma cell line, A704. Very few studies have shown such a comprehensive effect of EGF upon these sequential steps of the metastatic cascade. Therefore, we postulate that EGF may play an important role in enhancing aspects of tumour cell behaviour that may lead to increased metastatic propensity.

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