A role for transforming growth factor alpha and its receptor in human oesophageal cancer

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Abstract

A member of the epidermal growth factor (EGF) family, transforming growth factor alpha (TGF-α) shares significant homology with EGF and binds to the EGF receptor (EGF-R). Like EGF, TGF-α plays important roles in normal physiological processes; but, as its name signifies, it has potent transforming ability, often associated with autocrine stimulatory mechanisms. The purpose of this study is to investigate a possible role for TGF-α and its receptor in certain human oesophageal squamous cell carcinoma (SCC) cell lines - namely, WHCO-1, -3 and -5. The well-studied A431 epidermoid carcinoma cell line was used throughout for control purposes.

As shown by radioimmunoassay analysis, the subject cell lines, particularly WHCO-5, constitutively secrete appreciable quantities of TGF-α under unstimulated serum-free conditions. Exogenous phorbol 12-myristate 13-acetate (PMA), foetal bovine serum (FBS) and EGF augment TGF-α secretion to a statistically significant extent. The action of EGF is especially noteworthy because it indicates the presence of a functional autoinductive pathway. The high and low molecular weight species identified in the lysates of unstimulated cells by Western blot immunoprobe analysis are consistent with those reported in the literature. The former (meso-TGF-α) are of particular interest because, being cell-associated rather than secreted, they are suggestive of an autocrine mode of action. PMA and EGF, unlike FBS, appear to inhibit differential glycosylation. In the case of meso-TGF-α species, they may favour cleavage at the lys^{96}-lys^{97} cleavage site.

Cell proliferation and colony formation assays were carried out to determine the effect of exogenous TGF-α on the proliferative and

migratory behaviour of the subject cell lines. Their proliferative profiles exhibit a lack of uniformity that has frequently been reported elsewhere in respect of many derived cell lines. The WHCO-5 profile may be attributable to increasing down-regulation and desensitisation of the EGF-R. From the colony formation assays, it appears that exogenous TGF-α inhibits the formation of colonies in the A431 and WHCO-5 cell lines but promotes it in the WHCO-1 cell line. Regarding WHCO-3, exogenous TGF-α seems to promote migration because it yields fewer but larger and more diffuse colonies.

Hyperproduction of EGF-Rs has been described as the hallmark of SCCs. Overexpression of low-affinity EGF-Rs has recently been noted in a number of oesophageal SCC cell lines, including WHCO-1 and -3. In this study, ^{125}I-EGF competitive binding assays were performed to determine the hitherto unknown number and affinity of EGF-Rs in the WHCO-5 cell line. The values obtained, which indicate the presence of supernumary low-affinity receptors, are consistent with the consensus that emerges from earlier research.

The WHCO cell lines, especially WHCO-5, secrete relatively high levels of TGF-α; they also produce supernumary low-affinity EGF-Rs. Accordingly, dot-blot DNA hybridisation analyses were carried out to ascertain whether these phenomena are associated with amplification of the TGF-α and EGF-R genes. No evidence was found for TGF-α gene amplification, a result that is in agreement with the literature. On the other hand, amplification of the EGF-R gene was found to be a pronounced characteristic of the WHCO cells. This result is not surprising in the light of other research into primary oesophageal tumours and derived cell lines. A strong positive correlation was revealed between EGF-R gene amplification and protein overexpression in the WHCO cell lines.

There is extensive evidence in the literature that TGF-α interacts with the EGF-R by means of an autocrine pathway that is regulated in normal but deregulated in neoplastic cells, and in the latter confers a growth advantage that is important in tumour progression. Complete transformation typically requires more than one defect in signalling pathways. Hence, neoplastic cells may be expected to display overproduction of an autocrine mitogen coupled with a defect in one or more of the mechanisms which regulate the activity of its receptor. The experiments conducted in this study indicate that TGF-α and its receptor interact via an amplified autocrine pathway in certain oesophageal SCCs of the WHCO series. They not only constitutively produce and secrete large quantities of TGF-α but also concomitantly overexpress low-affinity EGF-Rs, characteristics that accord with research which suggests that TGF-α may be primarily dependent on this class of EGF-R for eliciting its mitogenic effect. Moreover, the seemingly novel identification of cell-associated meso-TGF-α is indicative of an autocrine (or intracrine) pathway. Finally, the ability of exogenous EGF to significantly enhance the accumulation of secreted TGF-α points to the existence of a functional autoinductive pathway. These findings support the conclusions concerning an autocrine role for TGF-α that have been reported in several other studies of oesophageal SCCs.

Declaration

I hereby declare that this dissertation ~~is my~~ own unaided work, unless otherwise indicated in the text. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

G.J. Jones

Gregory Justin Jones

..18.. day of January, 1993.

Preface

Several persons have contributed in varying degree to this study concerning the role of transforming growth factor alpha and its receptor in certain human oesophageal carcinoma cell lines.

To Dr. C.F. Albrecht of the Department of Pharmacology, University of Stellenbosch Medical School, I express my appreciation for his generous gift of the A431 cell line used throughout this study for control purposes.

My thanks are due to Dr. R. Derynck of the Department of Anatomy, University of California Medical School, who was the source of the TGF-α plasmid; and to Dr. M. Ramsey of the Department of Human Genetics, South African Institute for Medical Research, for kindly providing the β-globin plasmid.

The analysis of gene amplification was facilitated by Dr. E. Dabbs of the Department of Genetics, University of the Witwatersrand, who made helpful comments on the use of the TGF-α and EGF-R plasmids; and by Prof. N. Savage of the Department of Biochemistry, University of the Witwatersrand Medical School, who made available the Zeineh laser densitometer.

To Mrs. E. Scott and Ms. P. Manning of the Department of Zoology, University of the Witwatersrand, are due my thanks for their invaluable technical support.

Aspects of my research benefited from fruitful and friendly co-operation with Ms. N.S. Heiss, formerly of the University of the Witwatersrand, who was engaged in parallel studies of the role of cytoplasmic and

nuclear oncogenes in the subject and other cell lines.

To my father, Mr G.T.D. Jones, I express my appreciation for assistance with proof-reading the text and advice on statistical matters.

Above all, I wish to record an immense debt of gratitude to my supervisors, Prof. A.L. Thornley and Dr. R.B. Veale, both of the Department of Zoology, University of the Witwatersrand. This dissertation is the culmination of the friendly guidance and encouragement that they have afforded me over the years that I have been privileged to know them.

South African Biochemical Society Congress, 1992

At the eleventh congress of the Society, I presented a poster entitled: *In vitro* secretion of transforming growth factor alpha (TGF-α): A comparison of the A431 cell line with three human oesophageal squamous cell carcinoma cell lines. An abstract of the presentation appears at page 23 of the congress proceedings. Financial support for my attendance was provided by the University of the Witwatersrand.

Publications

Thornley, A.L. and Jones, G.J. (1992). *In vitro* secretion of transforming growth factor alpha (TGF-α): A comparison of the A431 cell line with three human oesophageal squamous cell carcinoma cell lines. *Bioscience Reports* 12, 293-302.

Jones, G.J., Heiss, N.S., Veale, R.B. and Thornley, A.L. (1992). Amplification and expression of the TGF-α, EGF receptor and c-*myc* genes in four human oesophageal squamous cell carcinoma cell lines. *South African Journal of Science*, to be submitted.

The material presented in the first of the aforementioned papers forms part of the subject-matter of Chapter 2. Data from the second paper have been used in Chapters 3 and 4.

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List of Abbreviations

Abbreviations for key recurrent terms, such as transforming growth factor alpha, are defined in the text when first used. Listed hereunder are the definitions of abbreviations that appear in the text relatively infrequently.

A	ampere
APS	ammonium persulphate
cDNA	complementary DNA
Ci	curie
Cs	caesium chloride
DAG	diacylglycerol
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
GAP	GTPase activating protein
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IgG	class G immunoglobulin
kb	kilobase
kd	kilodalton
M	molar
mol	mole
mRNA	messenger ribonucleic acid
OD	optical density
p	probability of error in rejecting null hypothesis
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute

SD	standard deviation
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TAE	tris, acetic acid, EDTA
TBE	tris, borate, EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	tris(Hydroxymethyl)aminomethane
V	volt

Chapter 1

General Introduction

1.1 Transforming growth factor alpha and other growth factors

Growth factors play key roles in both normal and aberrant cellular processes. Their many different types may be classified into five groups or families, as shown in Table I from Mercola and Stiles (1988). In general, each type binds to its cognate receptor in a highly localised, non-endocrine manner, thus stimulating the cell's biochemical circuitry. The combined action initiated by different kinds of ligand - sometimes co-operative, sometimes antagonistic - provides the fine tuning of relative proliferation and differentiation rates necessary for normal growth and development (Goustin *et al.*, 1986). Neoplasia is generated by dysfunctions in the signalling pathways (Massagué, 1985).

Transforming growth factor alpha (TGF-α), a member of the epidermal growth factor (EGF) family, is significantly homologous to EGF and binds to the EGF receptor (EGF-R). Like EGF, TGF-α is implicated in normal physiological processes such as embryonic induction (Cross and Dexter, 1991) and wound healing (Schultz *et al.*, 1991); but, as its name signifies, it has potent transforming ability, often associated with an autocrine stimulatory mechanism (see, for example, Kaplan *et al.*, 1982).

The aim of this study in broad terms is to present and discuss my research into the role of TGF-α and its receptor in certain human oesophageal squamous cell carcinoma (SCC) cell lines. This aim is

specified more precisely in section 1.4. First, however, it is necessary to develop a conceptual framework for two major themes that recur intermittently throughout the ensuing Chapters: namely, the multi-step nature of carcinogenesis and autocrine stimulation.

Table I Growth factor families

Group	Members
Epidermal growth factor	EGF TGF-α Vaccinia growth factor, VGF
Insulin-like growth factor	IGF-I IGF-II Relaxin
Transforming growth factor-β	TGF-β_1 TGF-β_2 TGF-$\beta_{1.2}$ Inhibin-A Inhibin-B Activin-A Activin AB Müllerian inhibiting substance
Heparin-binding growth factors	Acidic HBGF (acidic fibroblast growth factor, aFGF; endothelial cell growth factor, ECGF) Basic HBGF, bFGF Products of the *int*-2, *hst*, and Kaposi's Sarcoma pretooncogenes
Platelet-derived growth factor	PDGF-A PDGF-B (*sis* product) PDGF-AB

1.2 The multi-step nature of carcinogenesis

1.2.1 Cellular oncogenes

Weinberg (1991a), on which this section is largely based, argues that a human tumour is the end-point of a long process involving multiple changes in genotype and phenotype. According to one attractive model,

the cells in a tumour cell clone undergo a succession of genetic changes, each of which alters the cell genome in a way that yields advantageous growth phenotypes. Thus, the process that converts a normal cell into an aggressively growing agent of malignancy is based on the conversion of certain normal cellular genes (proto-oncogenes) into oncogenes.

Cellular oncogenes were first discovered through the study of retroviruses. A whole class of retroviruses - those capable of rapidly inducing tumours in infected host animals - were found to harbour transforming genes (viral oncogenes) whose origins were ultimately traced to similar genes residing in cellular genomes. The host cell genes that are the progenitors of these viral oncogenes presumably control essential aspects of normal cell physiology. The transducing ability of retroviruses enables them to use the host cell genes to transform infected cells. More than 60 cellular genes with oncogenic potential have now been identified (Bishop, 1991); some of the more important cognate oncogenes are listed in Table II, from Weinberg (1991a).

Retroviral transduction, however, is not the only mechanism by which specific cellular proto-oncogenes may become oncogenic. Transfection-transformation assays have proved that the same proto-oncogenes may also be activated in animals and humans by non-viral mechanisms involving somatic mutation. Point mutational activation of *ras* oncogenes leads to the synthesis of structurally altered Ras proteins; gene amplification has been implicated in the cases of *myc*, N-*myc* and *erb*B2/*neu*; and chromosomal translocations have been demonstrated in Burkitt's lymphoma.

1.2.2 Oncogenes and multi-step carcinogenesis

Successive genetic changes are necessary for a normal cell to become a cancer cell. For example, tumourigenesis in mice involves three stages. Firstly, tumour initiation can be achieved by a single mutagenic application of carcinogen, but the skin will remain normal indefinitely in the absence of further intervention. Tumour promotion ensues when a promoter, such as the phorbol ester phorbol 12-myristate 13-acetate (PMA), is applied. Although promoters are not mutagenic, their application to initiated cells results in papillomatous growths. These growths are pre-malignant, however, and disappear if promoter treatment is suspended. Tumour progression - the conversion of papillomas into autonomous growths - appears to require a second mutagenic event. Thus, it would seem that only doubly mutated cells develop into carcinomas.

It is not suggested, of course, that the above model describes the totality of the cancer process. Other changes occur that enable carcinoma cells to invade, to disseminate, to colonise distant sites as metastases and to evade immunological defences. These later events, too, are presumably driven by distinct changes in the tumour cell genome. Perhaps as many as half a dozen such changes may be involved in a cell clone's transition from normalcy to malignancy.

Tumourigenicity sometimes appears to require the co-operation of different oncogenes (Hunter, 1991). Rat embryo fibroblasts and kidney cells transfected by the *ras* oncogene are able to grow in an anchorage-independent manner in soft agar but unable to grow indefinitely in culture or to form tumours. Transfected by the *myc* oncogene, they become immortalised but are unable to grow in soft agar or to form

tumours. The cells become tumourigenic only when the two oncogenes are introduced concomitantly. These results indicate that a single type of oncogene, together with its encoded oncoprotein, is able to perturb only a part of the cell's regulatory circuitry. The fact that the *ras*-like oncogenes and the *myc*-like oncogenes encode cytoplasmic and nuclear proteins respectively suggests that there are distinct cytoplasmic and nuclear growth regulatory pathways that must be deregulated as a necessary, though probably not a sufficient, condition for complete cellular transformation.

Table II Examples of retrovirus-associated cellular oncogenes

Acronym	Species of origin	Retrovirus and tumour induced
src	Chicken	Rous sarcoma virus
yes	Chicken	Y73 sarcoma virus
ros	Chicken	UR2
mil	Chicken	MH2
jun	Chicken	AS17
myb	Chicken	Avian myeloblastosis virus
erb-A, *erb*-B	Chicken	Avian erythroblastosis virus
abl	Mouse	Abelson leukemia virus (pre-B cell lymphomas)
mos	Mouse	Moloney murine sarcoma virus
Ha-, Ki-*ras*	Rat	Harvey, Kirsten sarcoma virus
fos	Mouse	FBJ sarcoma virus
sis	Woolly monkey	Simian sarcoma virus
fms	Cat	FMS feline sarcoma virus
myc	Chicken	MC29 myelocytomatosis virus

Some tumours, however, carry only a *ras*-like or a *myc*-like oncogene, but not both. In these cases, it would appear that the presence of one or

other of the two types of oncogene coincides with the inactivation of a growth-suppressor gene (or anti-oncogene). The best-known of these is the *Rb* gene, whose inactivation triggers retinoblastomas and osteosarcomas. The evidence suggests that inactivation occurs only when both copies of the *Rb* gene on the paired chromosomes are disrupted.

Inactivation of an anti-oncogene is formally equivalent to activation of an oncogene. Thus, the co-operation necessary for tumourigenesis may involve the combined action of either *ras*-like and *myc*-like oncogenes or one of these coupled with the inactivation of an appropriate anti-oncogene.

1.2.3 Oncogenes and growth factor autonomy

The decision by a normal cell to proliferate, quiesce or differentiate seems to depend on exogenous stimuli, chiefly the mitogenic growth factors and growth inhibitory factors mentioned in Section 1.1 (Cross and Dexter, 1991). The neoplastic cell, by contrast, is characterised by independence from the intercellular signals that govern normal morphogenesis and maintain tissue structure. As noted in Weinberg (1991a), there are four established mechanisms by which oncogenes can induce cells to become independent of the exogenous growth factors normally required for their stimulation. These mechanisms are briefly described below.

1.2.3.1 Autocrine stimulation

Tumour cells often secrete mitogenic growth factors that stimulate them independently of exogenous growth factors. This may result from the alteration of growth factor genes so that they are expressed in an

unregulated, constitutive mode. Alternatively, and more frequently, oncogenes may constitutively turn on expression of the cell's growth factor genes. In point is the ability of the *ras*-encoded p21 oncoprotein to induce constitutive expression of TGF-α and other growth factors. These mechanisms are relevant to section 2.3.1 of Chapter 2.

1.2.3.2 Alterations in growth factor receptor molecules

As discussed in section 3.1.4 of Chapter 3, overexpression of the EGF-R may stimulate cellular proliferation in a context of reduced dependence on exogenous ligand. In addition, it is well known that aberrant receptors caused by structural alteration of receptor molecules may become constitutively active.

1.2.3.3 Alteration of cytoplasmic signalling pathways

The *ras* p21 and *src* p60 proteins, for example, receive and pass on excitatory signals from certain receptors on the cell surface. Their oncogenic forms, however, are constitutively active.

1.2.3.4 Alteration of nuclear signalling pathways

In the nucleus, which is the site of the ultimate cellular decisions, some of the nuclear genes inducible by growth factors are orchestrators of the action of a variety of subordinate genes. Their regulatory function can be disrupted. Thus, mutations of the *myc* proto-oncogene to an oncogene give rise to its constitutive expression and to consequent deregulation of cell growth.

Finally, it should be noted that activated growth-suppressor genes may play a similar role to that of activated oncogenes: they may induce

autonomous growth by making a tumour cell unresponsive to extracellular signals that would normally terminate its proliferation (Weinberg 1991b). One of the few examples of this mechanism known at present is a mutant allele of the thyroid hormone receptor gene: stem cells carrying this defective allele are unable to differentiate and thus may become exposed to the transforming potential of oncogenes such as *src* and *erb*-B.

1.3 The autocrine mechanism

1.3.1 Evidence of autocrine secretion

Mechanisms of growth regulation by polypeptides include endocrine, paracrine and autocrine systems. Factors, such as hormones, that enter the circulatory system and affect distant target cells are endocrine in nature. So is insulin-like growth factor, which is synthesised in the liver and affects distal tissues via the blood stream (Lyons and Moses, 1990). According to Ohlsson and Pfeifer-Ohlsson (1987), the stimulatory pathways of growth factors fall into two categories: paracrine (or endocrine/paracrine) and autocrine. In the paracrine model, the cell producing a relevant growth factor and the cell responding to the factor are phenotypically different and spatially separated; in the autocrine model, the cell produces both its own growth factors and its own receptors for those factors. The autocrine system is important in normal processes including embryogenesis, when paracrine mechanisms may be insufficiently developed; and it is clearly significant in malignant transformation by virtue of the growth advantage it confers.

The relatively autonomous nature of malignant cells, which require fewer exogenous growth factors for optimal growth than do their normal

counterparts, has been known for many years. Putative autocrine secretion of a growth factor in a cancer cell was first described in rodent cells transformed by Moloney or Kirsten murine sarcoma viruses (Mo-MSV and Ki-MSV respectively). The first peptides identified in connection with their activity are now known to have been TGF-αs, and the relationship between release of TGF-α and transformation has been well established by means of experiments with temperature-sensitive mutant rodent sarcoma viruses.

A representative early study is that of Kaplan *et al.* (1982). Temperature-sensitive mutants of Moloney and Kirsten sarcoma viruses were used to address the question whether TGF-α acts in an autocrine mode in virus-induced cell transformation. At the permissive temperature the cells were found to produce TGF-α, to have low serum requirements, to down-regulate the EGF-R and to be nonresponsive to exogenous EGF. At the non-permissive temperature they did not produce TGF-α and responded to EGF. These findings led Kaplan and his associates to conclude that growth factors produced by cells transformed by Ki-MSV can act ectopically and that the TGF-αs present are directly involved in reducing the serum requirement of the cells.

As noted by Sporn and Roberts (1991), numerous studies have now shown that the genes for various growth factors can be made to act as oncogenes under suitable experimental conditions, usually as part of a retrovirus construct that will allow overexpression of the growth factor in normal reader cells. Certain of these results suggest that autocrine stimulation of cell growth involving TGF-α may operate in the development of human neoplasia. In section 2.1.4 of Chapter 2, reference is made to a number of papers in which it has been quite rigorously shown that TGF-α acts as an autocrine growth factor in certain cancer cell lines, both *in vitro* and *in vivo*.

As previously mentioned, although the growth advantage conferred by autocrine secretion has obvious implications for carcinogenesis, the mechanism is not confined to cancer cells but is also important in normal cellular processes in adults as well as embryos. The fact that normal adult cells both produce and respond to TGF-α is strong evidence that not only transformed cells but also the proliferation of normal cells may be regulated in an autocrine manner (Lyons and Moses, 1990). A striking example is provided by Mead and Fausto (1989), who have shown that TGF-α appears to be an autocrine physiological regulator of liver regeneration and that its stimulatory effect on hepatocyte DNA synthesis is inhibited by a paracrine transforming growth factor beta (TGF-β) circuit, the balance of the two growth factors governing the regeneration process. Another example is the interaction of TGF-α and TGF-β in the modulation of mouse keratinocyte proliferation *in vitro* (Coffey *et al.*, 1988).

Knowledge of the autocrine mechanism has been extended and refined by recent research into autoinduction of growth factors, the role of nuclear transcription factors, intracrine and juxtacrine modes of stimulation, and the autocrine action of negative regulatory factors.

1.3.2 Autoinduction of growth factors

The ability of a growth factor to induce its own expression is a powerful mechanism for amplification of its autocrine action. This type of positive feedback was first demonstrated in the case of TGF-α (Coffey *et al.*, 1987) and was subsequently observed in several other growth factors (Coffey *et al.*, 1992). According to Klein *et al.* (1992), autoinduction of TGF-α in human keratinocytes occurs via a pathway that necessarily involves the tyrosine kinase of the EGF-R but does not appear to involve

downstream mediation by protein kinase C (PKC) activation. They speculate that autoinduction may be important in keratinocyte proliferation, whereas induction of TGF-α by PMA via a PKC-dependent pathway (Pittelkow *et al.*, 1989; Coffey *et al.*, 1992) may be involved in the regulation of keratinocyte differentiation. As will be explained in section 2.3.2 of Chapter 2, however, there is important evidence that PKC activation is implicated in autoinduction.

Autoinduction has now been observed in the context of a variety of normal and neoplastic cells. This finding lends support to the view that malignancy is probably the result of some deregulation of autocrine processes that occur, as shown for example by Mead and Fausto (1989), in normal cells. Nuclear transcription factors appear to be implicated in such deregulation.

1.3.3 The role of nuclear transcription factors

According to Sporn and Roberts (1991), recent studies indicate that nuclear transcription factors have an important role in controlling the functional activity of autocrine growth factors. For example, the Fos and Jun proteins interact to form a dimer with potent ability to stimulate specific gene transcription by binding at AP-1 sites on the promoter of target genes, thus enhancing RNA polymerase activity. It has been shown that they can regulate their own gene expression. It has also been demonstrated that TGF-β can up-regulate *jun* gene expression in a manner that involves an AP-1 binding site on the promoter. These findings point to the existence ot two autocrine loops that are mutually interactive. The loss of function of a necessary interface between the TGF-β and Fos-Jun systems may contribute to carcinogenesis.

For the purposes of the present study, it is of interest to note that TGF-α vigorously induces the expression of c-*fos* and c-*myc* in C3H 10T1/2 cells (Cutry *et al.*, 1988) and of c-*fos* in early post-implantation mouse embryos (Nielsen *et al.*, 1991). It may well be that the mechanisms involved are similar to those described in the preceding paragraph.

1.3.4 Intracellular autocrine stimulation

There is evidence that the mitogenic effects of a growth factor may be achieved not only by its secretion but also by its interaction with its receptor within some intracellular compartment, such as the Golgi apparatus or the endoplasmic reticulum. A schematic illustration of different possible mechanisms of autocrine growth stimulation is presented in Figure 1.1, which is taken from Heldin and Westermark (1989).

Intracrine stimulation (C in Figure 1.1) is a significant discovery because it implies that cells subject to it would be especially resistant to external regulation and have a particularly marked growth advantage. However, despite the fact that it has been demonstrated in laboratory experiments with defined cell lines and recombinant DNA constructs, intracrine stimulation has not yet been shown to be operative in human tumours (Sporn and Roberts, 1991).

1.3.5 Juxtacrine stimulation

Juxtacrine stimulation signifies communication between adjacent cells, membrane-bound growth factors on the one binding to receptors on the other. Studies with the TGF-α precursor have shown that, in its membrane-bound form, this molecule can establish contact with the

EGF-R of an adjoining cell and activate the receptor's tyrosine kinase activity (Wong *et al.*, 1989; Brachmann *et al.*, 1989). Anklesaria *et al.* (1990), to whom the term juxtacrine is due, have shown that this form of communication between TGF-α and the EGF-R can promote cellular adhesion and proliferation. It is possible that the juxtacrine ligand-receptor link permits reciprocal rather than conventional signal transduction (Pandiella and Massagué, 1991b).

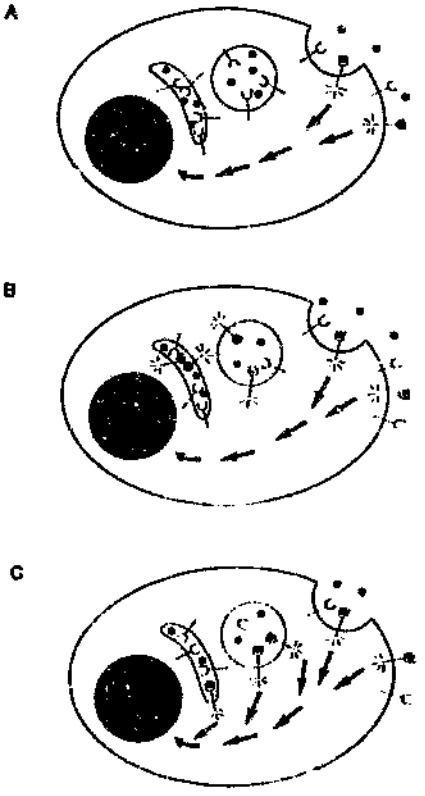


Figure 1.1 Schematic illustration of different possible mechanisms of autocrine growth stimulation. (A) The growth factor is secreted and activates its receptor at the cell surface only. (B) The growth factor activates its receptor also in the Golgi apparatus or in the secretory vesicles, but the ligand-receptor complex must be transported to the cell membrane in order to transduce the mitogenic signal further. (C) The growth factor activates its receptor and initiates all signals associated with the mitogenic response already in the Golgi apparatus or in the secretory vesicles.

1.3.6 Negative autocrine regulation

TGF-β is a potent inhibitor of DNA synthesis in almost all epithelial cells that are common sites of carcinogenesis, and also in haematopoetic precursor cells and T and B lymphocytes. It has a key role in the controlled processes by which normal cells replicate and differentiate (see, for example, Coffey *et al.*, 1988). Malignant transformation may ensue when cells become refractory to regulation by TGF-β for some reason, such as a biochemical lesion in the growth factor, its receptor or the post-receptor signalling pathway. Accordingly, there has been much research into the use of molecules in the steroid hormone family, such as retinoic acid and its analogues, as agents to stimulate the synthesis, secretion or activity of TGF-β.

As noted in Sporn and Roberts (1991), retinoic acid therapy has proved to be highly potent in the treatment of acute promyelocytic leukemia (APL). Studies of HL-60, a cell line derived from a patient with APL, have shown that retinoic acid induces synthesis of TGF-β_2 and the TGF-β receptor in these cells, which lack the receptor in their basal state. These studies suggest that retinoic acid acts to restore a negative or anti-proliferative autocrine loop to malignant cells. Tamoxifen, which stimulates TGF-β_1, appears to act similarly in MCF-7, a human breast cancer cell line. Retinoic acid is effective in the prevention of cancer in a variety of epithelia, and a clear relationship between it and stimulation of TGF-β_2 synthesis has been established in the case of skin cancer. In the latter case one may speculate that negative autocrine action is involved.

Although TGF-α has generally been observed in a positive autocrine mode, it is of interest to note that this peptide has recently been shown

to be a potent autocrine inhibitor of GH_4 pituitary tumour cell proliferation (Ramsdell, 1991).

1.4 Aim of the present study

This study is concerned with oesophageal cancer, which is the sixth most common cancer in the world (Cheng *et al.*, 1992). Carcinoma of the oesophagus is relatively common in certain parts of southern Africa (Bradshaw and Harington, 1987), although the reasons for its prevalence are a matter of speculation (Thornley *et al.*, 1991). In Black South Africans, oesophageal carcinoma has reached epidemic proportions since 1940, and at present is the most common cause of cancer-related deaths (Haffejee and Bryer, 1991).

As noted by Haffejee and Bryer (1991), SCCs constitute 95% of primary oesophageal malignancies. Oesophageal SCC is often far advanced at the time of its detection and as a result is generally associated with poor prognosis, especially if it is correlated with gastric involvement by lymph node and intramural metastases (Kuwano *et al.*, 1992). The epidemiology, aetiology, pathology and current treatment of this carcinoma have been described in Haffejee and Bryer (1991).

The specific aim of the present study is to report and discuss the findings of my research into the possiblity that TGF-α acts in a positive autocrine mode in three human oesophageal SCC cell lines: namely, WHCO-1, -3 and -5, the abbreviation WHCO signifying Witwatersrand University human carcinoma of the oesophagus. These lines were established from biopsies taken from middle-aged Black patients during routine investigations of the oesophagus. The biopsies were pathologically characterised as having a moderate degree of keratinocyte

differentiation and a squamous cell infiltration of the tissues underlying the oesophageal epithelium. Their establishment procedures, DNA profiles and histological characteristics have been described in Thornley *et al.* (1991).

In Chapter 2, I discuss my experimental results regarding the production, secretion and biological action of TGF-α in the WHCO-1, -3 and -5 cell lines. In Chapter 3, I report on the first experimental findings regarding the hyperproduction and affinity of EGF-Rs in WHCO-5 cells, which may be linked with their relatively high levels of TGF-α secretion. Chapter 4 is concerned with a possible role for gene amplification in overexpression of TGF-α and EGF-Rs in the subject cell lines. The conclusions that emerge from this study are set out in Chapter 5, together with some thoughts on the direction of future research.

Chapter 2

Transforming Growth Factor Alpha

2.1 Introduction

2.1.1 Biological background

The discovery of transforming growth factors arose from the observation that retrovirus-transformed rat kidney cells had a marked deficiency of EGF-Rs on their surfaces, suggesting that such cells must release an EGF-like factor able to compete with EGF for binding sites. It was thought that this factor had been identified in the medium of certain retrovirus-transformed murine fibroblasts. It was assumed initially that the factor was a single peptide (De Larco and Todaro, 1978) but subsequent analysis showed that two structurally unrelated peptides - TGF-α and TGF-β - were involved. Their generic name, transforming growth factor, stems from the fact that they are able to reversibly transform immortalised normal rat kidney fibroblasts in soft agar assays (Todaro *et al.*, 1980). Although binding to EGF-Rs is due solely to the presence of TGF-α, the profound morphological changes manifested by these cells are now known to be caused by co-operative interaction between TGF-α and TGF-β (Anzano *et al.*, 1983).

2.1.2 Structure of TGF-α and its precursor

Secreted TGF-α exists as a family of species, ranging in apparent size from 6kd to 20kd. Species with apparent molecular weights of more than

20kd have, however, been identified (see, for example, Luetteke *et al.*, 1988; Van de Vijver *et al.*, 1991). The mature and smallest form is a 6kd species comprising 50 amino acids, as shown in Figure 2.1 from Tam *et al.* (1991).

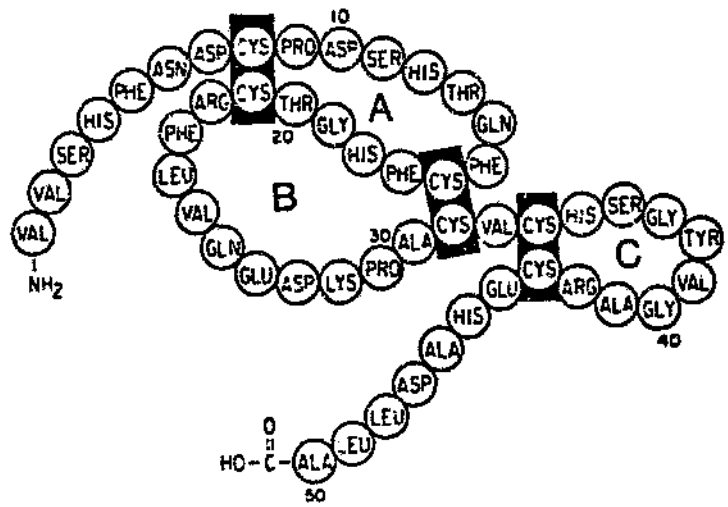


Figure 2.1 Amino acid sequence of mature human TGF-α. Solid boxes represent disulphide bridges.

Mature TGF-α is about 30% homologous with EGF and this homology, together with the three similar disulphide bridges in both EGF and TGF-α, provides a molecular explanation of the fact that both bind to the EGF-R (Derynck, 1988). Analysis of the solution structure of TGF-α by means of nuclear magnetic resonance spectroscopy and site-directed mutagenesis studies has led to the identification of the residues that contribute to its complementary EGF-R binding surface (Tam *et al.*, 1991; Field *et al.*, 1992). As shown in Figure 2.2 from Tam *et al.* (1991), these residues are clustered at one end of the TGF-α molecule. This cluster is composed of three discontinuous fragments: namely, the phe[15]-

cys-leu tripeptide of loop A; the arg[42]-cys-glu tripeptide of loop C; and the asp[47]-leu dipeptide of the external carboxyl sequence.

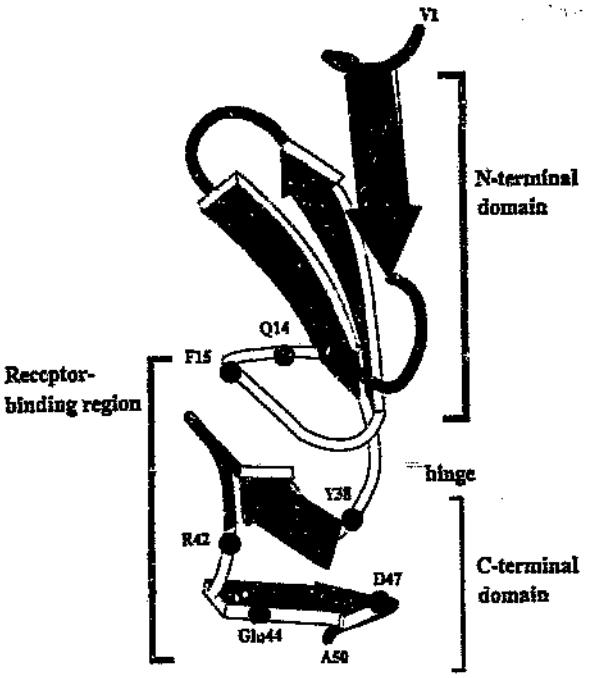


Figure 2.2 Proposed receptor-recognition site of mature human TGF-α. It consists of part of the A-loop [phe[15](F15)-cys-leu] and C-loop [arg[42](R42)-cys-glu], as well as the carboxyl terminal peptide sequence [asp[47](D47)-leu].

cDNA characterisation has revealed that mature TGF-α is synthesised as an internal part of a transmembrane glycoprotein precursor, designated pro-TGF-α (Texeidó and Massagué, 1988), that is 160 amino acids long in humans (Derynck *et al.*, 1984). The precursor contains an extracellular domain of about 100 amino acids, including the amino-terminal signal sequence, the N- and O-glycosylation sites and the mature TGF-α; an extremely hydrophobic transmembrane domain of 23 amino acids; and a cysteine-rich cytoplasmic domain of 35 amino acids (Bringman *et al.*, 1987; Teixidó *et al.*, 1987; Massagué, 1990). The integral membrane glycoprotein properties of the precursor have been

verified using metabolic labelling experiments (Bringman *et al.*, 1987), membrane-associated mRNA translation techniques (Teixidó *et al.*, 1987) and biochemical and immunocytochemical procedures involving transfected and tumour-derived cell lines (Wong *et al.*, 1989; Brachmann *et al.*, 1989). The human TGF-α precursor is depicted in Figure 2.3.

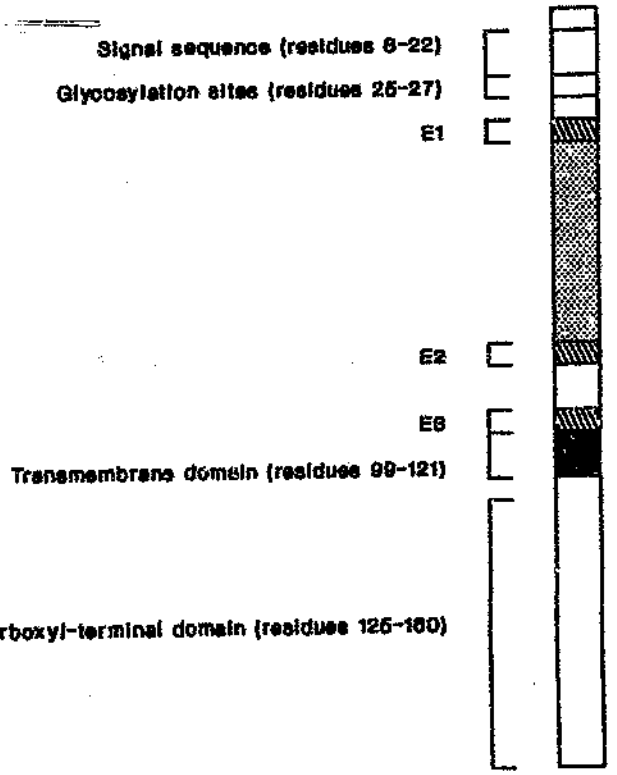


Figure 2.3 Schematic representation of the human TGF-α precursor (based on Bringman *et al.*, 1987). The N- and O-glycosylation sites occur within the asn-ser-thr sequence beginning at residue 25 (Luetteke *et al.*, 1988); E1 represents the ala^{39}-val^{40} cleavage site; the stippled box, mature TGF-α; E2 the ala^{89}-val^{90} cleavage site; and E3 the dibasic lys^{96}-lys^{97} cleavage site.

2.1.3 TGF-α's maturation and processing pathway

Pro-TGF-α is translated from a 4.5 to 4.8kb mRNA (Derynck, 1987) as an 18kd nascent polypeptide (Wong *et al.*, 1989). Translation of pro-TGF-α *in vitro* in the presence of microsomal membrane vesicles has shown that this polypeptide is translocated across the endoplasmic reticulum by means of its amino terminal signal sequence (Teixidó *et al.*, 1987). Translocation is arrested at the hydrophobic transmembrane spanning domain. Nascent pro-TGF-α increases in mass following post-translational modifications; carbohydrate units are added within the endoplasmic reticulum and Golgi apparatus to the N- and O-glycosylation sites (Teixido *et al.*, 1987; Pandiella and Massagué, 1991a), and palmitate side chains are linked to cysteine residues in the carboxyl terminus (Bringman *et al.*, 1987). In several cell types, the extent of N-linked carbohydrate modification varies, giving rise to a heterogeneously glycosylated product (Teixidó and Massagué, 1988). In Chinese hamster ovary (CHO) cells, N- and O-glycosylation are not required for the expression of pro-TGF-α on the cell surface or the generation of soluble TGF-α (Bringman *et al*, 1987; Teixidó *et al.*, 1990). In retrovirally transformed rat fibroblasts, however, inhibition of N-glycosylation interferes with TGF-α production (Teixidó and Massagué, 1988).

The release of mature TGF-α from the outer domain of human pro-TGF-α occurs following proteolytic cleavage at sequences on the carboxyl side of alanine residues 39 and 89 (Derynck *et al.*, 1984). The extracellular or cell-surface proteinases responsible for this cleavage are presumed to have elastase-like activity (Teixidó *et al.*, 1990) but have not yet been identified (Choudry and Kenny, 1991). Cleavage at lysine residues 96 and 97 by an enzyme with trypsin-like activity may also play a contributory role in some cell types (Bringman *et al.*, 1987; Teixidó and

Massagué, 1988). The proteolytic process that releases mature TGF-α is inefficient under basal conditions in most cultured cell lines (Teixidó *et al.*, 1990) and may either lead to the release of partially processed forms (Luetteke *et al.*, 1988; Teixidó and Massagué, 1988; Teixidó *et al.*, 1990), collectively designated meso-TGF-α (Teixidó and Massagué, 1988), or to the accumulation of pro-TGF-α on the plasma membrane (Teixidó *et al.*, 1990; Brachmann *et al.*, 1989). Pro-TGF-α exposed on the surface of one cell can interact with EGF-Rs on the surface of adjacent cells and thereby effect cell-cell adhesion (Anklesaria *et al.*, 1990), receptor autophosphorylation (Brachmann *et al.*, 1989; Wong *et al.*, 1989), transmembrane signalling (Wong *et al.*, 1989) and mitogenesis (Anklesaria *et al.*, 1990).

Teixidó and Massagué (1988) have outlined a processing pathway in connection with a study of retrovirally transformed rat embryo fibroblasts. It was found that these cells release to the medium not only mature TGF-α but also a group of meso-TGF-αs of 17-20kd. The latter were shown to correspond to the heterogeneously glycosylated extracellular domain of pro-TGF-α. As may be seen from Figure 2.4, the processing of pro-TGF-α in transfected rat embryo fibroblasts appears to involve two stages. The first leads to a meso-TGF-α by elastase-mediated cleavage at the ala^{88}-val^{89} site, possibly preceded by trypsin-mediated cleavage at the lys^{95}-lys^{96} site. The second leads to a mature TGF-α by elastase-mediated cleavage of meso-TGF-α at the alanine-valine site located in the amino terminus.

This processing pathway appears also to be operative in the chemically-induced rat hepatocellular carcinoma cell line JM1 (Luetteke *et al.*, 1988) but not in CHO cells transfected with a rat pro-TGF-α cDNA sequence. In the latter, cleavage at the ala^{88}-val^{89} site is a rate-limiting step in the generation of soluble TGF-α and is preceded by the more

rapid cleavage at ala[38]-val[39] (Teixidó *et al.*, 1990). This cleavage-rate discrepancy is surprising, given the similarity of the N- and C-terminal cleavage sequences, and is not understood (Massagué, 1990). [In passing, it is pointed out that cleavage sites are shifted by one amino acid residue towards the amino terminus in rat pro-TGF-α, which has a length of 159 amino acids (Lee *et al.*, 1985) compared with 160 amino acids for humans].

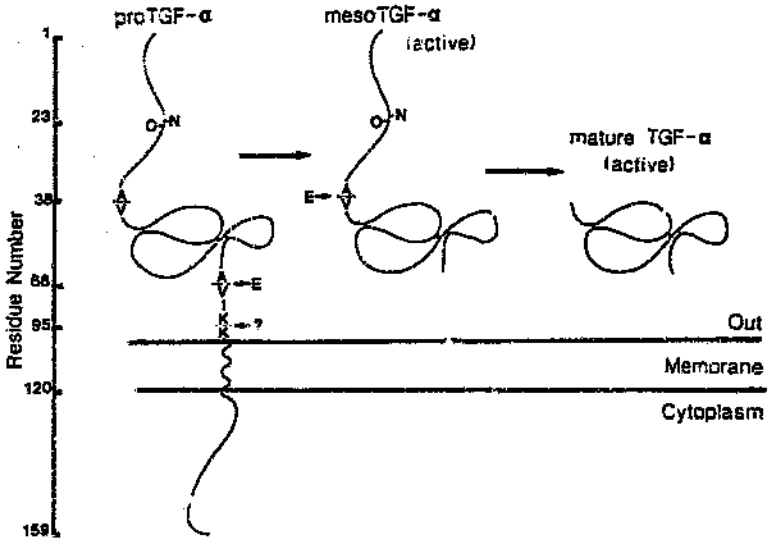


Figure 2.4 The conversion of membrane-bound pro-TGF-α into soluble, bioactive products. Rat pro-TGF-α is a 159-amino acid protein with a hydrophobic sequence between residues 97 and 120 that acts as a membrane anchor. The NH_2-terminal domain faces the extracellular side of the membrane, and contains carbohydrate N-linked to asparagine 25 (-N), and O-linked carbohydrate (-O). The extracellular domain includes the 50-amino acid sequence corresponding to mature TGF-α. This sequence is flanked by alanine-valine sequences (A/V). An elastase-like activity (E) cleaves at the A/V site in the COOH terminus of the mature TGF-α sequence. As a result, the 17-19kd heterogeneously glycosylated meso-TGF-α is released into the medium as a soluble, bioactive molecule. Further cleavage of this intermediate precursor at the A/V site located in the NH_2 terminus of the TGF-α sequence yields mature TGF-α. A lysine-lysine (K/K) sequence in position 95-96 of pro-TGF-α might serve as an additional site for cleavage and release of the extracellular domain.

Recent work on transfected CHO cells has shown that the cleavage of

pro-TGF-α is a highly regulated process that may be acutely and rapidly enhanced by a number of exogenous agents (Pandiella and Massagué, 1991a and b). The calcium ionophore A23187 enhances cleavage by a mechanism that depends on the influx of extracellular calcium and is largely independent of PKC. PMA stimulates cleavage via PKC and does not require extracellular calcium. Serum factors enhance cleavage by a mechanism that is largely independent of both PKC and the influx of extracellular calcium. These three mechanisms may either affect distinct components of the cleavage process, involving either the enzyme or its substrate, or converge on a common component of the cascade of events that activate cleavage (Pandiella and Massagué, 1991b). As noted in Pandiella and Massagué (1991c), the regulation of pro-TGF-α cleavage represents an interesting mechanism for the release of hormonally active factors from a cell. In addition to serving as an outlet for soluble TGF-α, stimulation of pro-TGF-α cleavage terminates the function of this molecule as a mediator of intercellular communication by cell-cell contact, and could also terminate the as yet hypothetical role of pro-TGF-α as a signalling receptor.

2.1.4 The biological activity of TGF-α

Expression of TGF-α has been observed in a wide variety of normal cells. During embryonic development in the mouse and rat, TGF-α is transiently synthesised in several tissues, including the placenta, the uterus, the developing kidney and central nervous system, the nasopharyngeal pouch, and the optic vesicle (Derynck, 1988; Tamada *et al.*, 1991; Lazar and Blum, 1992). TGF-α mRNA has recently been detected in the human placenta (Bissonnette *et al.*, 1992), the foetal kidney (Goodyer *et al.*, 1991) and the foetal pancreas (Miettinen and Heikinheimo, 1992) and its protein implicated in autocrine stimulatory

mechanisms. The TGF-α gene is also expressed in adult animals. TGF-α is synthesised by normal skin keratinocytes (Coffey *et al.*, 1987), by activated macrophages (Madtes *et al.*, 1988; Rappolee *et al.*, 1988) and by eosinophils (Wong *et al.*, 1990; Todd *et al.*, 1991). In cultured keratinocytes, TGF-α has been shown to stimulate its own expression. This process of autoinduction has been proposed as a mechanism of signal amplification which may be essential for the propagation of proliferative responses throughout an organ or tissue (Lyons and Moses, 1990). Furthermore, TGF-α mRNA is present in pituitary cells (Samsoondar *et al.*, 1986), rat liver cells (Mead and Fausto, 1989; Evarts *et al.*, 1992) and in the adult mammalian brain (Kudlow *et al.*, 1989; Seroogy *et al.*, 1991). In the rat liver, TGF-α plays an important role in regeneration (Mead and Fausto, 1989) and in hepatic cell migration both *in vitro* and *in vivo* (Bade and Fiendler, 1988; Evarts *et al.*, 1992). The presence of TGF-α in the brain, a non-proliferative tissue, is surprising and little understood. Although the brain cells may not proliferate in response to oncogene induction or growth factor stimulation, neurons and glial cells throughout the brain exhibit EGF-Rs and therefore TGF-α may act as a signalling peptide within the brain (see, for example, Ojeda *et al.*, 1990).

The foregoing suggests that TGF-α has important roles in normal physiology - including the promotion of embryonic processes, wound healing and organ regeneration, and certain signalling roles in the brain. In addition to these normal roles, however, TGF-α is critically implicated in the process of malignant transformation, the context of its discovery and the origin of its name.

Expression of the TGF-α gene has been demonstrated in a variety of tumours, chiefly in carcinomas, but not as yet in haematopoetic cell lines. The consistent expression of the TGF-α gene in SCCs and renal

carcinomas is frequently accompanied by elevated levels of EGF-R expression (Derynck *et al.*, 1987). As noted in Karnes *et al.* (1992), possibly the most convincing evidence supporting a direct role for TGF-α in malignancy comes from experiments in which unregulated expression of TGF-α in transgenic mice results in malignant changes in breast and hepatic epithelia.

Although the autocrine stimulatory mechanism is involved in both normal and neoplastic processes, it is perhaps particularly significant in the latter. Addition of TGF-α to MDA-MB-468 breast cancer cells leads to increased levels of EGF-R mRNA (Fernandez-Pol *et al.*, 1989). Furthermore, addition of EGF or phorbol esters to these cells results in the concurrent accumulation of TGF-α and EGF-R mRNA, thus promoting autocrine growth (Bjorge *et al.*, 1989). An autocrine function for TGF-α in malignancy has been rigorously demonstrated in many cell lines. These include lines derived from ovarian adenocarcinomas (Morishige *et al.*, 1991; Kurachi *et al.*, 1991); androgen-independent prostatic carcinomas (Hofer *et al.*, 1991); and, which is of particular relevance to the present study, oesophageal SCCs (Yoshida *et al.*, 1990; Reiss *et al.*, 1991). These studies all indicate that TGF-α and its receptor act in an autocrine mode *in vitro*; the paper by Kurachi *et al.* (1991) shows that they can act similarly *in vivo*. Because human cancer results from multiple genetic lesions, it is likely that growth factor autocrine loops act synergistically with other molecular perturbations in the pathogenesis and progression of the disease (Stromberg *et al.*, 1992).

2.1.5 Comparative biological activity of TGF-α and EGF

Given that TGF-α and EGF both bind to the EGF-R, the question arises

whether the two growth factors are functionally equivalent.

It should be noted that comparative studies have focused on EGF and mature TGF-α (Derynck, 1988). The two growth factors have similar activity in some assays. For example, they are about equivalent in their ability to stimulate DNA synthesis in various cell lines, to induce anchorage independence (in the presence of serum and TGF-β) in immortalised rodent fibroblasts, and to induce eyelid opening in newborn mice.

In other assays, however, EGF and TGF-α have been found to give rise to different responses. In most of these, TGF-α appears to have greater potency than EGF, although some other differences may be qualitative. Thus, TGF-α seems to have a greater ability than EGF to cause membrane ruffling, its effects being enhanced by TGF-β, whereas that of EGF is reduced (Myrdal *et al.*, 1986). TGF-α is more active than EGF in the induction of monolayer colony formation of epidermal cells, an event that in turn depends on cell migration and proliferation (Barrandon and Green, 1987). Both EGF and TGF-α can induce neovascularisation in an *in vivo* assay, but TGF-α is the more potent (Schreiber *et al.*, 1986). TGF-α is also more potent than EGF in the release of Ca^{2+} from bones in culture (Ibbotson *et al.*, 1986).

It appears, then, that TGF-α is a superagonist of EGF in several systems, but that in others it may have qualitatively distinct activities. Certain arguments have been put forward to explain these differences. As noted in Lewis *et al.* (1990), it is possible that these growth factors interact with their common receptor in different ways or that they might cause different conformational changes in the receptor. It has been established that the two ligand-receptor complexes are processed differently within the cell (Decker, 1990; Ebner and Derynck, 1991).

Furthermore, the possibility of a distinct TGF-α receptor in some cell types cannot be excluded (see, for example, Chalazonitis *et al.*, 1992).

2.1.6 Aim of Chapter 2

This Chapter has a twofold aim. Firstly, I propose to investigate the production and secretion of TGF-α by A431, WHCO-1, -3 and -5 cells under unstimulated and stimulated conditions, using Western blot and radioimmunoassay analyses. Secondly, I explore the biological action of exogenous mature TGF-α on the subject cell lines using proliferation and colony formation assays.

2.2 Materials and Methods

2.2.1 Routine cell culture

The A431, WHCO-1, -3 and -5 SCC cell lines were grown in surface culture at 37°C in a humid atmosphere of 5% CO_2 and 95% air. The lines were maintained on basal cell culture medium comprising a three to one mixture of Dulbecco's modified Eagles medium and Ham's F12 (DMEM/F12), plus 10% foetal bovine serum (FBS, Hyclone). Cell cultures were fed twice a week and passaged prior to confluence using 0.5% trypsin/0.1% EDTA in sterile phosphate buffered saline (PBS, pH 7.6; see section A1 of Appendix A).

2.2.2 Radioimmunoassay for secreted TGF-α

2.2.2.1 Collection of conditioned media

Basal cell culture media were aspirated and the cell monolayers washed

with PBS. After a 4 hour preincubation in DMEM/F12, which was then removed, 10ml of fresh DMEM/F12 were added to each culture dish and left for conditioning at 37°C over a period of 20 hours. In those cultures that received stimulatory agents, EGF (10ng/ml, Sigma) was added at the beginning of the 20 hour conditioning period (Coffey *et al.*, 1987; Pittelkow *et al.*, 1989), and PMA (500nM, Sigma) and FBS (10%, Hyclone) were added 15 minutes prior to the end of this period (Pandiella and Massagué, 1991a and b). The conditioned media were later assayed for TGF-α content to [illegible]s the levels of unstimulated and stimulated secretion per million cells. The average numbers of viable cells present at the beginning and at the end of the conditioning period were quantified haemocytometrically by trypan blue exclusion (Anklesaria *et al.*, 1990).

All conditioned media were collected and clarified by centrifugation in the presence of 0.02ml 1M HEPES plus 0.036ml 0.028M PMSF per ml conditioned medium and stored at -20°C. With regard to the procedure described in Hanauske *et al.* (1987), conditioned medium samples were dialysed at 4°C against 1% acetic acid using 3.5kd cut-off dialysis membrane (Spectra/Por) and then lyophilised. Dialysed samples were subsequently reconstituted in tris-buffered saline (TBS, pH 8.5; Biomedical Technologies Inc.) to a final concentration of 10 times for use in a radioimmunoassay.

2.2.2.2 Radioimmunoassay and statistical analysis

The experiment was designed to determine whether the chosen cell lines secrete TGF-α under unstimulated and stimulated conditions and, if so, whether the differences between the levels of the latter and the former were statistically significant. The quantity of TGF-α present in the medium conditioned by unstimulated and stimulated cells was

determined using a sensitive and highly specific radioimmunoassy kit (Biomedical Technologies Inc.) according to the manufacturer's instructions (see section A2.1 of Appendix A). Using standard curves with recombinant TGF-α, experimental and control ^{125}I-TGF-α gamma counts were expressed as picagram equivalents of secreted TGF-α per million cells (see section A2.2 of Appendix A). The standard curves were linear for TGF-α concentrations between 0 and 0.30ng/ml, the range in which the experimental logα values were observed. Linear regression equations based on this range had extremely high coefficients of correlation and very small standard errors. Non-specific binding was less than 4% in all cases.

Statistical analysis was directed towards determining whether mean experimental gamma counts differed from those of the pertinent controls in a directionally significant (one-tailed) sense at a 5% (that is $p \leq 0.05$) level of confidence or better. The parametric Student t test for independent samples was used for this purpose where the variance-ratio F test permitted, failing which the corresponding Mann-Whitney U test (Siegel, 1956) was used. In all cases it was found that the results were significant at a 5% level of confidence or better. One-tailed tests were deemed to be valid because, on theoretical grounds, one could predict that mean experimental gamma counts would be lower than those of the pertinent controls. In virtually all cases, however, the results are statistically significant at the 5% level under a two-tailed test.

2.2.3 Western blot and immunoprobe analysis of endogenous TGF-α

2.2.3.1 Cell lysate preparation

Unstimulated and stimulated cell monolayers were briefly rinsed with PBS, detached by scraping in detachment solution (see section A3 of Appendix A) and transferred to 10ml centrifuge tubes. The cells were pelleted by centrifugation for five minutes at 1000 rpm in an MSE bench-top centriguge. After aspirating the detachment solution, 1ml of electrophoresis sample buffer (see section A4 of Appendix A) was added to each tube and the cell pellets were dispersed by vortexing. The cells were lysed by boiling the cell mix for 5 minutes and the lysates stored at -20°C until required.

2.2.3.2 Protein electrophoresis and electroelution

Proteins within the cell lysate samples were separated by electrophoresis at 28mA and 8°C in denaturing 10-20% gradient SDS polyacrylamide gels (see sections A5.1 and A5.2 of Appendix A) according to the method of Laemmli (1970). The proteins were then transferred to nitrocellulose sheets (0.45 micron Hybond-C, Amersham) by electroelution in transfer buffer (see section A5.3 of Appendix A) at 400mA and 5°C for approximately 75 minutes (Towbin *et al.*, 1979). Low molecular weight markers (Pharmacia) were used for calibration purposes.

2.2.3.3 Immunoprobe analysis

The electroeluted nitrocellulose sheets were immersed in blocking buffer

(see section A6 of Appendix A) for 1 hour and then incubated for between 2 and 4 hours in an anti-human TGF-α primary antibody solution. The nitrocellulose sheets were subsequently incubated in a horse-radish peroxidase-conjugated antibody solution for 1 to 2 hours. Before and after the antibody incubations the sheets were washed for 2x10 minutes in TBS Tween- 20 (TBST, see section A7 of Appendix A). The primary and secondary antibodies used, which are described in detail in section A8 of Appendix A, were diluted in TBST. Finally, the nitrocellulose sheets were developed in a 4-chloro-1-napthol substrate solution (see section A9 of Appendix A).

2.2.4 In vitro effects of exogenous TGF-α

2.2.4.1 Cell proliferation assays

The methodology followed was based on that described in Markowitz *et al.* (1990) and Chen *et al.* (1991). Cells were plated out into 24 well tissue culture plates (Nunc) at a density of $5x10^4$ cells/well in DMEM/F12 plus 5% FBS. After approximately 18 hours, the medium was aspirated and replaced with 1ml/well of DMEM/F12. Twenty four hours later, this medium was removed and replaced with 0.5ml/well of DMEM/F12 containing 0.1 to 100ng/ml of recombinant mature human TGF-α (Sigma). Control points received 0.5ml/well DMEM/F12 without any growth factor supplement. The number of viable cells was quantified haemocytometrically by trypan blue exclusion 48 hours after growth factor addition.

2.2.4.2 Colony formation assays

Cells were plated out in 6cm tissue culture dishes (Nunc) at a density

of 10^3 cells/dish in DMEM/F12 plus 5% FBS. After colonies had developed to a size of approximately 15 cells, 10ng/ml of TGF-α (Sigma) was added to the "experimental" dishes but not to their controls. Eight days after this step, the medium was aspirated and the dishes briefly rinsed with PBS. The cells were then fixed, using formal-PBS (4% formaldehyde in PBS), and the colonies stained with PAGE-blue biological stain.

2.3 Results and Discussion

2.3.1 Constitutive production and secretion of TGF-α

Constitutive secretion of TGF-α has been demonstrated in numerous cancer cell lines, including those derived from ovarian carcinomas (Stromberg *et al.*, 1992) and colon adenocarcinomas (Karnes *et al.*, 1992). Secretion of TGF-α by the A431 epidermoid carcinoma cell line, used here as a positive control, has been reported by a number of researchers (Derynck *et al.*, 1987; Reiss *et al.*, 1991; Van de Vijver *et al.*, 1991). However, a recent study by Cardinali *et al.* (1992) failed to detect soluble TGF-α in the medium conditioned by these cells over a period of 72 hours. A surprising finding of this study was the ability of suramin, a chemotherapeutic agent, to stimulate secretion markedly. A recent report by Reiss *et al.* (1991) has demonstrated that constitutive secretion of TGF-α is a common characteristic of SCC cell lines, including the A431 cell line and, which is of particular interest in the context of the present study, one derived from an oesophageal tumour.

My results show that, under unstimulated serum-free conditions, the A431, WHCO-1, -3 and -5 SCC cell lines constitutively secrete significant

quantities of -α over a period of 20 hours (see Table III). Such secretion may be attributable to the mechanisms referred to in section 1.2.3.1 of Chapter 1. It is important to note that the measured level of secreted TGF-α may underestimate the endogenous production of this ligand because it can exist, as noted in section 2.1.3, as a membrane-bound biologically active precursor. Yoshida *et al.* (1990) have recently demonstrated that certain oesophageal carcinoma cell lines express as much as 38 to 60.2 picagrams per million cells of cell surface pro-TGF-α.

Table III Quantity of TGF-α secreted in 20 hours under unstimulated serum-free conditions

Cell line	No. cells[1]	ng TGF-α/ml[2]	pg TGF-α/10^6 cells[3]
A431	$1.75x10^7$ $2.05x10^7$	0.032 0.037	3.6 3.6
WHCO-1	$1.96x10^7$	0.053	5.4
WHCO-3	$5.53x10^6$ $1.03x10^6$ $1.86x10^7$	0.024 0.048 0.078	8.6 9.7 8.4
WHCO-5	$9.80x10^6$	0.072	146.0

1 Conditioning 20ml of DMEM/F12.
2 Radioimmunoassay sensitivity = 0.02ng TGF-α per tube.
3 Values are significant at $p \leq 0.05$ and represent the average of duplicate determinations from a single experiment.

Expressed on a pg/10^6 cell basis, the quantity of TGF-α secreted by WHCO-5 in 20 hours is approximately 41 times that secreted by A431 cells over the same time period (present study) and some 5-19 times that secreted by certain other cancer cell lines in 48 hours (Derynck *et al.*, 1987; Smith *et al.*, 1987; Kim *et al.*, 1991). Although to the best of my knowledge no comprehensive survey of TGF-α secretion levels has been published to date, it appears that the quantity of TGF-α secreted by WHCO-5 cells under unstimulated serum-free conditions is relatively high for a neoplastic cell line. The reason for this is not known at

present. Amplification of the TGF-α gene, enhanced expression and stabilisation of its mRNA and unusually efficient cleavage of its protein product could have played contributory roles; the first of these is explored in Chapter 4. In contrast to most of the WHCO cell lines reported on elsewhere, WHCO-5 has an unusually short doubling time (approximately 20 hours; Prof. A.L. Thornley, personal communication). The *in vitro* growth behaviour of this cell line may, at least in part, be the result of a signalling defect involving overexpression of TGF-α and the EGF-R. It is possible, however, that this behaviour, compared with that of the WHCO-1 and -3 cell lines, is also partly attributable to a relative dysfunction in the action of some factor, possibly TGF-β, that inhibits cell growth in these cell lines. As mentioned in section 1.3.1 of Chapter 1, TGF-β frequently has an important role in counterbalancing the autocrine stimulatory effects of TGF-α.

Cell lysate samples obtained from the subject cell lines were electrophoresed on 10-20% gradient polyacrylamide gels and electroeluted to nitrocellulose sheets. The sheets were then subjected to immunoprobe analyses, the results of which are shown in Figure 2.5.

In panel a of Figure 2.5, two distinct forms of TGF-α were found to react specifically with a sheep polyclonal anti-human TGF-α antiserum (Biomedical Technologies Inc.). The lower molecular weight form (approximately 21kd) is consistent with that reported in the literature for O-glycosylated pro-TGF-α (Wong *et al.*, 1989); the higher weight form (approximately 37kd) falls within the wide range recorded for glycosylated meso-TGF-α species (Luetteke *et al.*, 1988). The greater intensity of the bands for WHCO-5 reflects the fact that this cell line produces relatively more TGF-α.

Panel b of Figure 2.5 shows the results of immunoprobe analysis of

lysates from the A431 and WHCO-3 cell lines using a mouse monoclonal anti-TGF-α antibody (Oncogene Science). The two higher molecular weight species (34kd and 37kd) fall within the acceptable range for differentially glycosylated meso-TGF-α (Luetteke *et al.*, 1988; Van de Vijver *et al.*, 1991). It is conceivable, however, that the difference in the meso-TGF-α molecular weights is partly attributable to differential cleavage at the ala^{89}-val^{90} and lys^{96}-lys^{97} cleavage sites (Bringman *et al.*, 1987). The lower molecular weight species accord with forms of pro-TGF-α, the 21.4kd form being O-glycosylated (Wong *et al.*, 1989) and the 18kd form lacking carbohydrate moieties (Teixidó *et al.*, 1990).

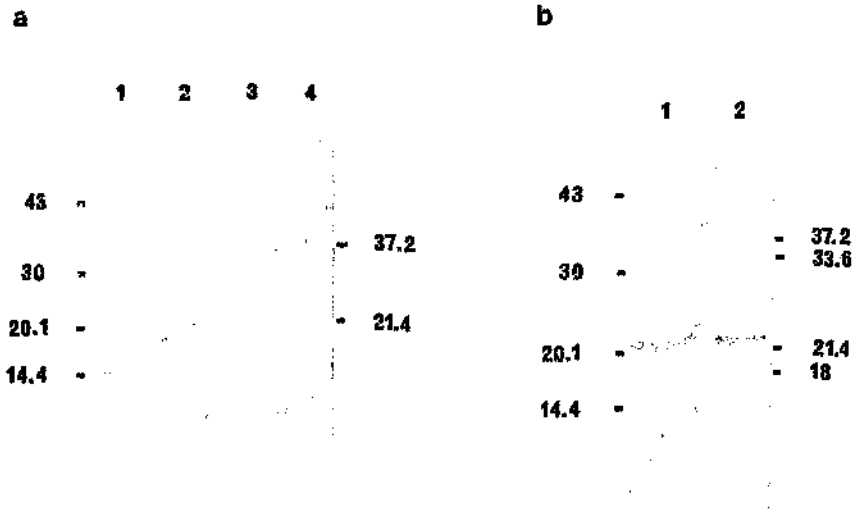


Figure 2.5 Biosynthesis and processing of TGF-α under unstimulated serum-free conditions. Panel **a** - Western immunoblot of cell lysate samples from A431 (lane 1), WHCO-1 (lane 2), WHCO-3 (lane 3) and WHCO-5 (lane 4) cells cultured for 20 hours under unstimulated serum-free conditions. Twenty five microlitres of lysate sample (containing approximately 1.02×10^4 cells/μl) were loaded in each lane. The primary immunoprobe was a highly specific polyclonal sheep antiserum raised against mature human TGF-α (Biomedical Technologies Inc.). Panel **b** - Western immunoblot of A431 (lane 1) and WHCO-3 (lane 2) cell lysate samples (25μl per lane). The primary immunoprobe was a mouse monoclonal antibody raised against recombinant human TGF-α (residues 33-50, Oncogene Science). In both panels, the positions and molecular weights (kd) of the protein standards are indicated on the left, and the weights of the immunoreactive TGF-α bands on the right.

The upper molecular weight bands in Figure 2.5 are of particular interest because they represent forms of TGF-α that have elsewhere been found to be secreted (see, for example, Luetteke *et al.*, 1988 and Van de Vijver *et al.*, 1991), but here are cell-associated. One possible explanation is that meso-TGF-α may act in an autocrine mode, binding to its receptor after short range diffusion. This speculation is not precluded by the degradation of internalised ligand-receptor complexes because, as shown by Ebner and Derynck (1991), a significant portion of internalised TGF-α is recycled to the cell surface in undegraded form. Further support for this speculation is provided by Van de Vijver *et al.* (1991), who have shown that in A431 cells species of meso-TGF-α of at least 30kd become cell-associated via an autocrine pathway. My results may also be explicable in terms of intracrine stimulation, in which meso-TGF-α species are generated intracellularly and act on EGF-Rs in the Golgi apparatus or endoplasmic reticulum (see Figure 1.1, Chapter 1). Both intracrine and autocrine mechanisms may be operative: Van de Vijver *et al.* (1991) assign a minor role to the former in A431 cells, whereas Bringman *et al.* (1987) ascribe a minor role to the latter in transfected CHO cells.

2.3.2 Agent-stimulated secretion of TGF-α

Many studies, some of which are cited below, have shown that secretion of TGF-α by normal and neoplastic cells may be enhanced above basal levels following stimulation with specific agents. These include PMA, serum factors, EGF and TGF-α. The purpose of this section is to report on the efficacy of PMA, FBS and EGF in promoting TGF-α secretion in the subject cell lines.

PMA enhances the secretion of TGF-α in cultured epidermal

keratinocytes (Pittelkow *et al.*, 1989; Klein *et al.*, 1992), in transfected CHO cells (Pandiella and Massagué 1991a and b), and in HT1080 fibrosarcoma cells (Pandiella and Massagué 1991a). My results demonstrate that the secretion of TGF-α from A431 and WHCO-3 cells is significantly and rapidly (within 15 minutes) elevated above basal levels following exposure to 500nM PMA (see Figure 2.6).

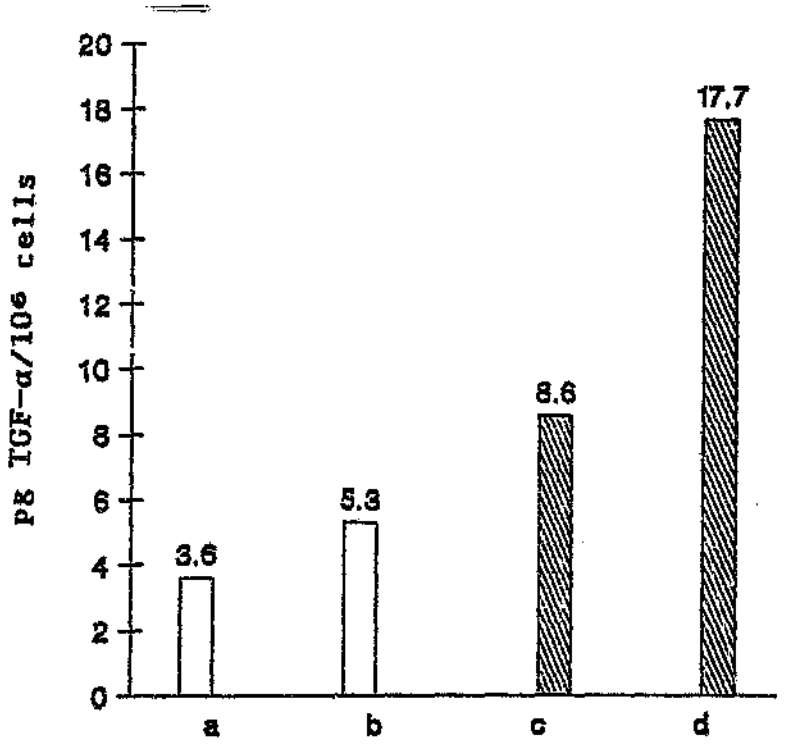


Figure 2.6 PMA-stimulated secretion of TGF-α under serum-free conditions. **a** Total TGF-α secreted by unstimulated A431 cells in 20 hours, **b** total TGF-α secreted by PMA-stimulated A431 cells, **c** total TGF-α secreted by unstimulated WHCO-3 cells in 20 hours, **d** total TGF-α secreted by PMA-stimulated WHCO-3 cells. PMA was added to a final concentration of 500nM 15 minutes prior to the end of the 20 hour conditioning period. Secretion values are significant at $p \leq 0.05$ and represent the average of duplicate determinations from a single experiment.

At this concentration, PMA increases the rate of TGF-α secretion 38-fold

in A431 cells and 84-fold in WHCO-3 cells. Similar findings have been reported in PMA-stimulated transfected CHO cells (Pandiella and Massagué, 1991a). The mechanism by which PMA enhances TGF-α secretion in these cells may depend on PKC signalling, which has been shown to be operative in transfected CHO cells (Pandiella and Massagué, 1991a). Although the activation of PKC can result in the elevated expression of a variety of genes (Pittelkow *et al.*, 1989; Pandiella and Massagué, 1991a), including the gene for TGF-α (Bjorge *et al.*, 1989; Anklesaria *et al.*, 1990), the rapid nature of the observed cleavage response suggests that this PKC-dependent mechanism does not rely upon the relatively time-consuming synthesis of pro-TGF-α but rather upon activation of an essential cleavage enzyme, increased exposure of pro-TGF-α to this enzyme, or conversion of pro-TGF-α to a better substrate for the enzyme.

FBS has been found to enhance the secretion of TGF-α by a number of human tumour cell lines (Derynck *et al.*, 1987). In this study I have demonstrated that the secretion of TGF-α from A431 and WHCO-3 cells is appreciably and quickly (within 15 minutes) raised above basal levels following exposure to 10% FBS (see Figure 2.7). Comparable findings have recently been reported in serum-stimulated transfected CHO cells, in which serum factors acutely activate pro-TGF-α cleavage via PKC-dependent and -independent mechanisms (Pandiella and Massagué, 1991a and b). The cleavage-stimulating factor present in FBS is not a protease and is probably of platelet origin (Pandiella and Massagué, 1991b).

Finally, I consider the ability of EGF to enhance the secretion of TGF-α. EGF stimulates the secretion of TGF-α protein in cultured epidermal keratinocytes (Coffey *et al.*, 1987; Pittelkow *et al.*, 1989) and in certain melanoma and carcinoma cells (Singletary *et al.*, 1990). It should be

noted that in cultured epidermal keratinocytes TGF-α has been shown to induce its own expression by means of an autoinductive mechanism that presumably sustains and may amplify cellular responses to this growth factor (Coffey *et al.*, 1987). Here I show that a 20 hour exposure to 10ng/ml EGF significantly and autoinductively increases the accumulation of secreted TGF-α in the media conditioned by A431, WHCO-1, -3 and -5 cells (see Table IV).

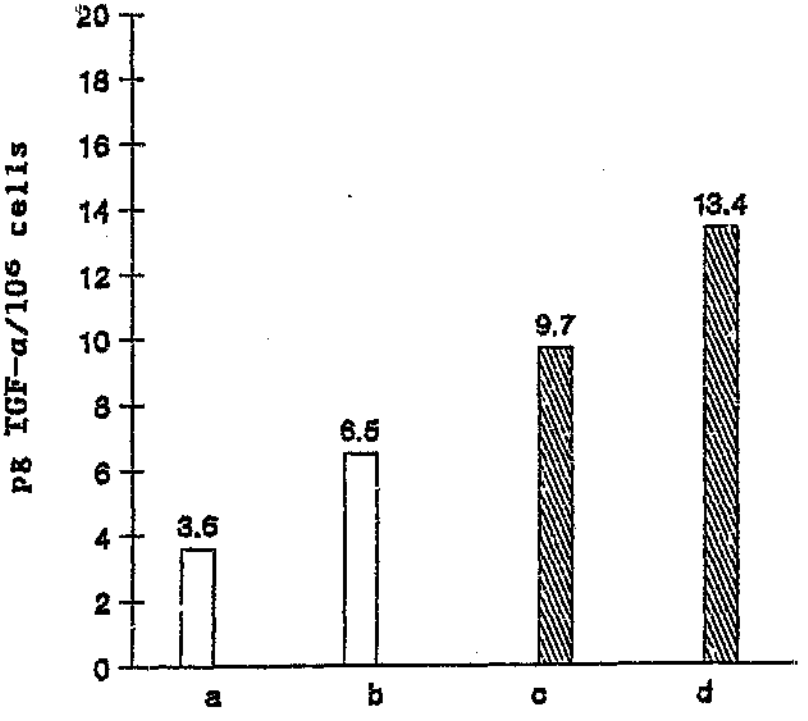


Figure 2.7 FBS-stimulated secretion of TGF-α. **a** Total TGF-α secreted by unstimulated A431 cells in 20 hours, **b** total TGF-α secreted by FBS-stimulated A431 cells, **c** total TGF-α secreted by unstimulated WHCO-3 cells in 20 hours, **d** total TGF-α secreted by FBS-stimulated WHCO-3 cells. FBS was added to a final concentration of 10% 15 minutes prior to the end of the 20 hour conditioning period. Secretion values are significant at $p \leq 0.05$ and represent the average of duplicate determinations from a single experiment.

Table IV Quantity of TGF-α secreted in 20 hours under EGF-stimulated serum-free conditions.

Cell line	TGF-α secreted[1] in presence of EGF[2]	TGF-α secreted[1] in absence of EGF	Enhancement factor[3]
A431	12.5	3.6	2.5
WHCO-1	6.4	5.4	0.2
WHCO-3	11.8	8.4	0.4
WHCO-5	1448.6	146.0	8.9

1 pg/10^5 cells.
2 10ng/ml.
3 Enhancement factor = (column 2-column 3)/column 3.

The enhancement of TGF-α secretion is most pronounced in the case of WHCO-5 cells. These results necessarily involve the relatively lengthy process of EGF-enhanced TGF-α gene expression (Coffey *et al.*, 1987; Pittelkow *et al.*, 1989) but also imply an increased cleavage efficiency, which may well be linked to PKC activation. As noted in section 1.3.2 of Chapter 1, there is some evidence that autoinduction requires not only EGF-R tyrosine kinase activation but also subsequent PKC signalling triggered by the former (Pittelkow *et al.*, 1989; Coffey *et al.*, 1992). The triggering mechanism involves phosphorylation of phospholipase C-γ_1 (PLC-γ_1) which, in turn, generates diacylglycerol (DAG) to activate PKC. Thus, DAG, the endogenous activator of PKC, may operate to increase cleavage efficiency in much the same way as the exogenous PKC activator PMA.

Figure 2.8 shows the result of an immunoprobe analysis of A431 and WHCO-3 whole cell lysate samples obtained from unstimulated cells and from cells stimulated by various agents as specified in the legend. Oncogene Science's mouse monoclonal antibody was used as the primary immunoprobe.

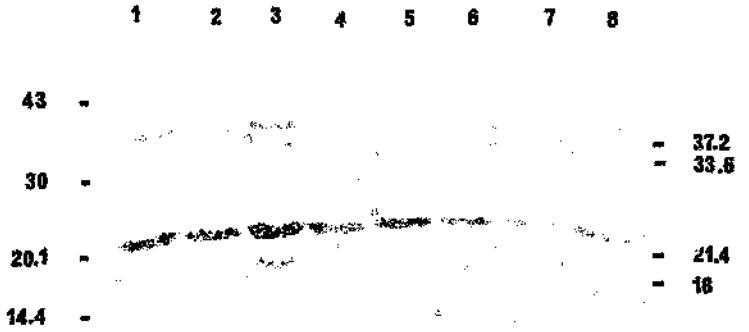


Figure 2.8 Biosynthesis and processing of TGF-α in A431 and WHCO-3 cells under unstimulated and stimulated conditions. Western immunoblot of cell lysate samples from A431 cells - unstimulated (lane 1), PMA-stimulated (lane 2), FBS-stimulated (lane 3), EGF-stimulated (lane 4); and from WHCO-3 cells -unstimulated (lane 5), PMA-stimulated (lane 6), FBS-stimulated (lane 7), EGF-stimulated (lane 8). The concentration and duration of application of the exogenous stimulatory agents are specified in section 2.2.2.1. Twenty five microlitres of lysate sample (containing approximately $1.02x10^4$ cells per μl) were loaded in each lane. The primary immunoprobe was Oncogene Science's mouse monoclonal anti-human TGF-α antibody. The positions and molecular weights (kd) of the protein standards are indicated on the left, and the weights (kd) of the immunoreactive TGF-α bands on the right.

The low and high molecular weight forms for WHCO-3 correspond to glycosylated pro-TGF-α and meso-TGF-α respectively. One would expect the lower band of lane 5 to be more intense than those in lanes 6 and 7, and less intense than that in lane 8; and the upper bands of lanes 6 to 8 to be more intense than that of lane 5. In both cases, however, intensity appears to be independent of stimulation. The reasons for this lack of variation are not understood, but in the case of the upper bands it may conceivably result from saturation of cell surface EGF-Rs by meso-TGF-αs.

As regards A431, Figure 2.8 depicts high and low molecular weight forms of TGF-α that are comparable with those of WHCO-3. No variation in intensity is observable although the expected relative intensities of the four lanes are the same as those in the preceding paragraph. The lack of variation of intensity in the lower bands may again be attributable to receptor saturation.

It is of interest to note that the presumed differential glycosylation of unstimulated A431 cells (lane 1) also occurs following FBS stimulation (lane 3) but only minimally after stimulation by PMA and EGF (lanes 2 and 4 respectively), suggesting that the latter pair of stimulatory agents inhibit such glycosylation. As regards the lower molecular weight forms of meso-TGF-α, the hypothesis of differential proteolytic cleavage (section 2.3.1) would suggest that PMA and EGF, unlike FBS, may favour cleavage at the lys^{96}-lys^{97} cleavage site.

2.3.3 In vitro effects of exogenous TGF-α

Thus far in Chapter 2, it has been shown that the subject cell lines constitutively produce and secrete varying quantities of TGF-α under unstimulated serum-free conditions, and that secretion can be significantly stimulated by various exogenous agents. As will be shown in Chapter 3, they also overexpress the EGF-R. In the light of these facts it is appropriate to consider the effects of exogenous mature TGF-α on the proliferation and migration of these cell lines *in vitro*. Accordingly, cell proliferation and colony formation assays were undertaken on the bases set out in section 2.2.4. The results are discussed below.

2.3.3.1 Cell proliferation assays

Graphs of the cell proliferation assays are presented in Figure 2.9. In each panel, the lower horizontal line represents the mean number of cells unexposed to exogenous TGF-α. The upper horizontal line represents this mean plus one standard deviation (SD). The irregular top line shows the trend of mean cell numbers as a function of increasing concentration of exogenous TGF-α; SDs of plus one and minus one are also indicated. In each case, the concentration of exogenous TGF-α ranges from 0 to 100 ng/ml.

Panel a shows that the proliferation of A431 cells is stimulated above the base level by exogenous TGF-α in the range of 0.1 to 10ng/ml, but thereafter declines towards the base line. I am not aware of comparable studies involving TGF-α, but the results reported for its homologue, EGF, are naturally of interest. Cowley *et al.* (1984) have shown that, under similar conditions, EGF in the range of 0.1 to 3.0ng/ml stimulates the proliferation of A431 cells but thereafter progressively inhibits it to levels that ultimately fall below control levels. This led them to suggest that, compared with normal keratinocytes, the observed responsiveness of A431 cells to low levels of EGF might be attributable to their overexpression of EGF-Rs. Reiss *et al.* (1991) show progressive enhancement of DNA synthesis - and possibly, therefore, of proliferation - within the range they considered (i.e. 0 to 10ng/ml). Noteworthy is their caveat to the effect that the responsiveness of A431 cells to exogenous EGF-R ligands is highly variable, possibly because of the use of different sublines with distinct phenotypes.

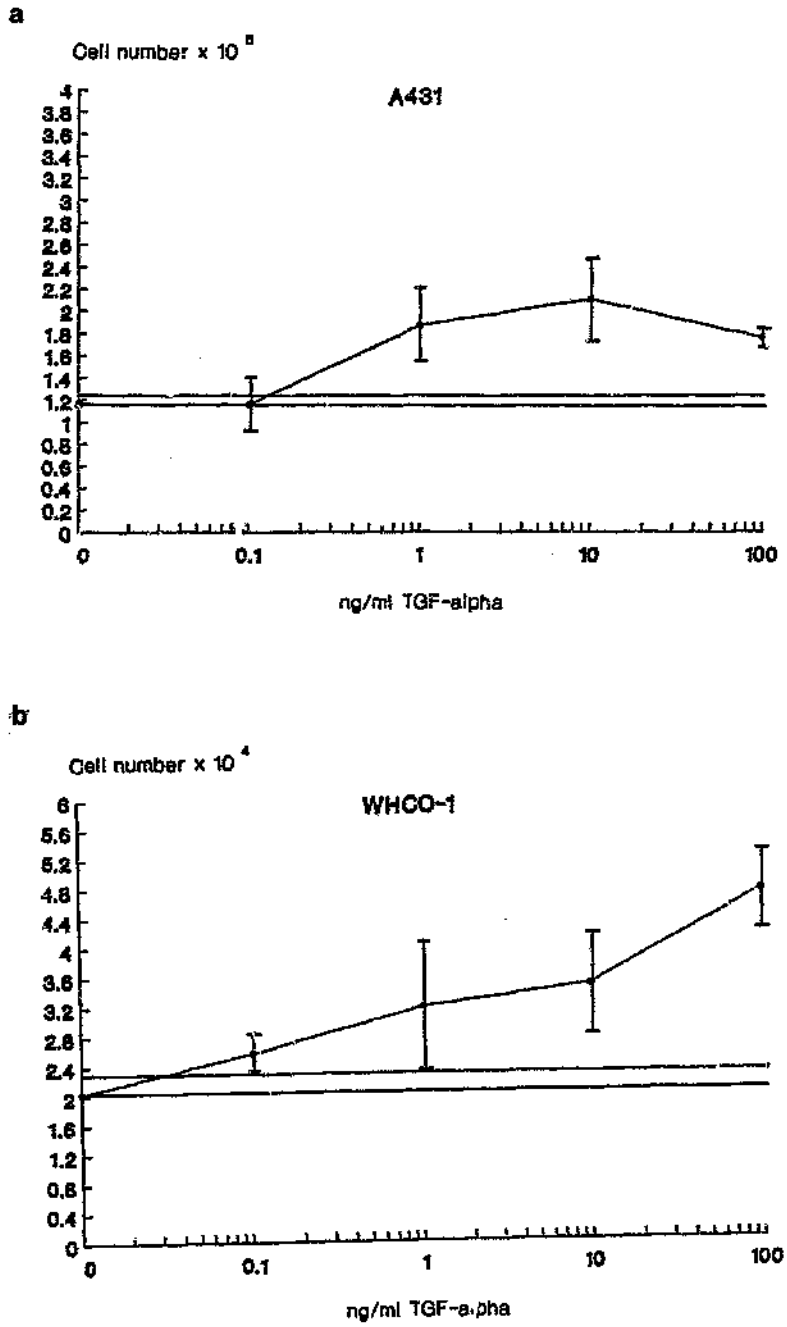
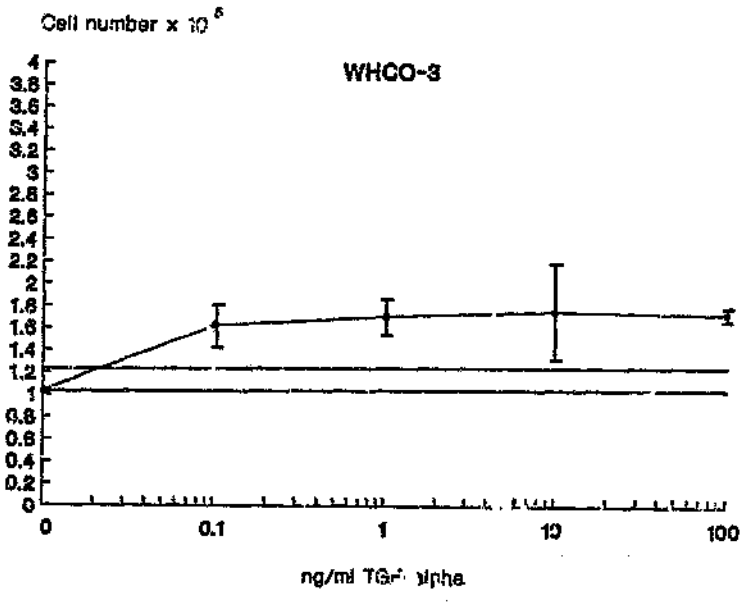


Figure 2.9 Cell proliferation assays. Panel a, A431; panel b, WHCO-1. Cells were plated out at a density of $5x10^4$ cells/well in 24 well plates in 1ml DMEM/F12 plus 5% FBS. Eighteen hours after this step, the medium was aspirated and 1ml of DMEM/F12 was added to each well. Twenty four hours later (day 0), the medium was removed and replaced with 0.5ml/well DMEM/F12 containing 0 to 100 ng/ml TGF-α. Viable cell numbers were determined haemacytometrically on day 2 by trypan blue exclusion. Data, from a single experiment, represent the mean of three to four points ± one SD.

c



d

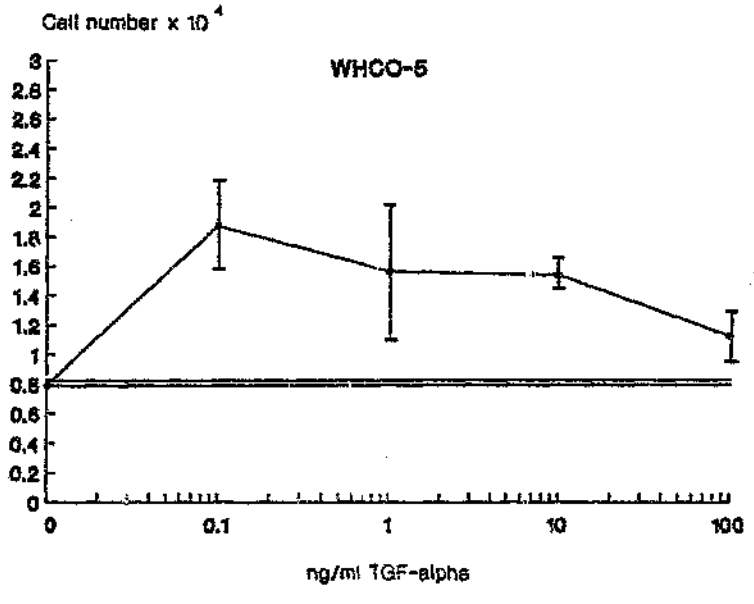


Figure 2.9 (continued) Cell proliferation assays. Panel c, WHCO-3; and panel d, WHCO-5. Cells were plated out at a density of $5x10^4$ cells/well in 24 well plates in 1ml DMEM/F12 plus 5% FBS. Eighteen hours after this step, the medium was aspirated and 1ml of DMEM/F12 was added to each well. Twenty four hours later (day 0), the medium was removed and replaced with 0.5ml/well DMEM/F12 containing 0 to 100 ng/ml TGF-α. Viable cell numbers were determined haemacytometrically on day 2 by trypan blue exclusion. Data, from a single experiment, represent the mean of three to four points ± one SD.

From panel b, it may be seen that the proliferation of WHCO-1 cells increases progressively with the addition of exogenous TGF-α to approximately 2.4 times basal level at a TGF-α concentration of 100ng/ml. In this connection, it is of interest to note that Chen *et al.* (1991), in a study of the CE-48 human oesophageal SCC cell line, showed that the addition of exogenous TGF-α resulted in a similarly dose-dependent enhancement of proliferation to 4.8 times the basal level at a concentration of 600ng/ml.

The results for panel c show that the proliferation of WHCO-3 cells is moderately responsive to exogenous TGF-α in the range of 0 to 0.1ng/ml, but thereafter is unaffected by increasing ligand concentration. A possible explanation is advanced in the next section.

Finally, I consider the proliferative pattern of the WHCO-5 cell line. As is apparent from panel d, proliferation is enhanced to a maximum level of 2.4 fold at 0.1ng/ml and then progressively declines, closely approaching the base level at 100ng/ml. Over the relevant range, the pattern is similar to that reported by Stromberg *et al.* (1992) for the OVCA 420 ovarian carcinoma cell line. I would speculate that in the WHCO-5 cell line the decline in proliferation from 0.1ng/ml onwards may be attributable to increasing down-regulation and desensitisation of the EGF-R. This speculation is consistent with the fact that this cell line secretes relatively high amounts of TGF-α under unstimulated serum-free conditions (see section 2.3.1) and that this secretion is greatly augmented by exogenous EGF (see section 2.3.2).

2.3.3.2 Colony formation assays

The colony formation assays are depicted in Figure 2.10. The culture dishes on the left hand (panel a) are controls that show colony formation

in the absence of exogenous TGF-α for the A431, WHCO-1, -3 and -5 cell lines respectively; those on the right hand (panel b) show colony formation in the corresponding cell lines in the presence of 10ng/ml of exogenous TGF-α. The number of colonies per culture dish is tabulated in Table V.

Table V Colony formation assays - colonies per culture dish.

Cell line	Number of colonies per dish (no TGF-α)	Number of colonies per dish (10ng/ml TGF-α)
A431	281	124
WHCO-1	77	102
WHCO-3	215	114
WHCO-5	65	31

The first (horizontal) pair of dishes shows that in A431 cells the addition of exogenous TGF-α in serum-containing medium inhibits colony formation, a result that accords with the findings for this cell line by Banks-Schlegel and Quintero (1986) in relation to exogenous EGF, and by Kumar and Mendelsohn (1990) in relation to exogenous TGF-α. A similar degree of inhibition is manifested in WHCO-5. In contrast, the addition of TGF-α to the WHCO-1 cell line promotes colony formation. The reasons for this discrepant behaviour are not understood, but are not surprising in the light of Veale and Thornley (1989). As noted in their paper, there is no relationship between the biological response to EGF, which is homologous to TGF-α, and the number of EGF-Rs per cell type, which is necessarily implicated in colony formation. Indeed, variability of cell proliferation profiles appears to be the rule rather than the exception (see, for example, Stromberg *et al.*, 1992).

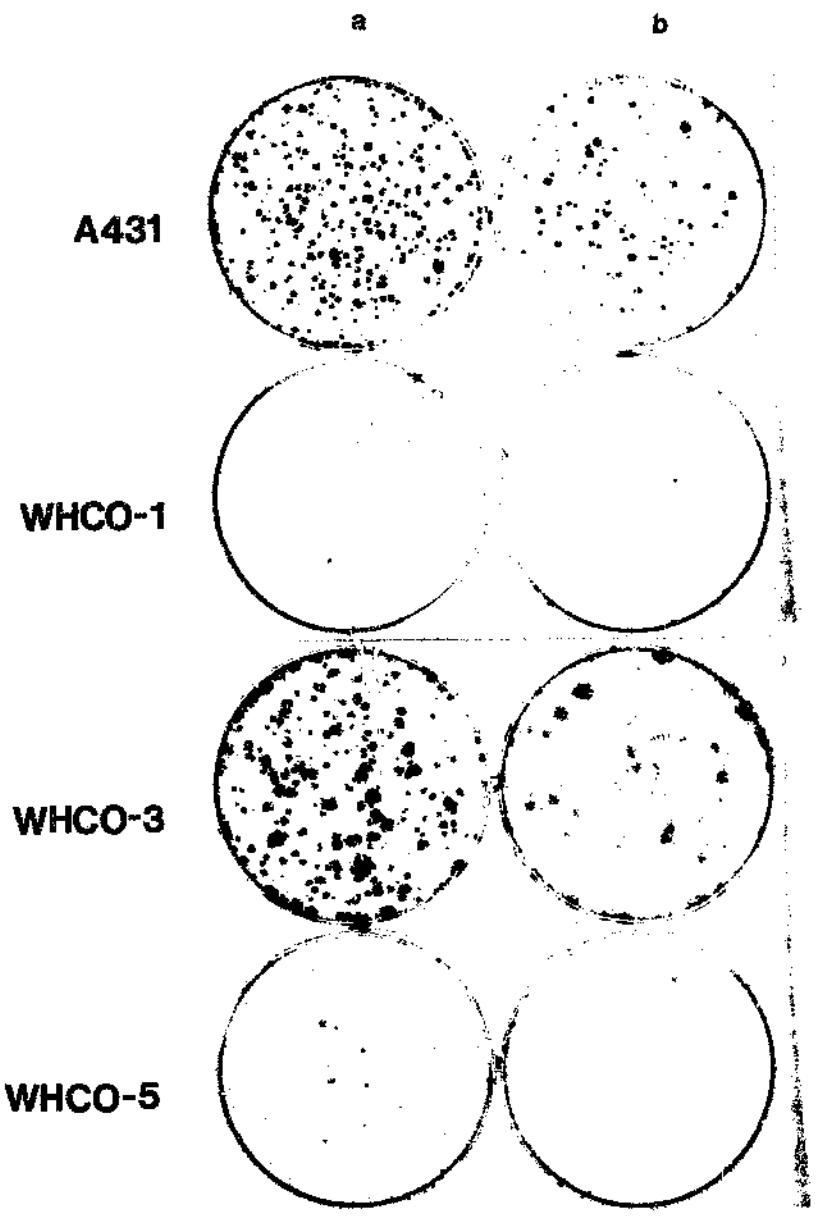


Figure 2.10 Colony formation assays. Cell lines are shown on the left. Panel **a** - no exogenous TGF-α, panel **b** - 10ng/ml exogenous TGF-α. Cells were plated out at a density of 10^2 cells/dish in DMEM/F12 plus 5% FBS. Growth factor was added after colonies had developed to a size of approximately 15 cells (day 0). Cells were fixed and colonies stained on day 8.

Inspection of the third pair of dishes, containing WHCO-3 cells, shows that the right hand dish contains fewer (see Table V), but larger and more diffuse, colonies than the left hand or control dish. This suggests that the addition of 10ng/ml of exogenous TGF-α resulted in cell migration and coalescence into larger colonies. Thus, the plateau observed in panel c of Figure 2.9 may mean that, in the range of 0.1 to 100ng/ml, exogenous TGF-α sustains proliferation of WHCO-3 cells by promoting their migration.

It is appropriate at this point to comment on the mechanisms that link proliferation and migration. In Barrandon and Green (1987), it has been shown that the growth of an epidermal keratinocyte colony depends on outward migration of proliferating cells at the colony perimeter. Such growth is sustained by exogenous EGF and TGF-α, in the absence of which the rate of cell migration becomes a limiting factor and cells in the interior of the colony tend to leave the basal layer and terminally differentiate. EGF has been shown to stimulate migration in a number of normal and neoplastic cells. This effect, which is important for the maintenance of proliferative potential in normal keratinocytes and for the process of wound healing, is brought about by alteration in the expression of certain cytoskeletal and secretory protein genes. Migratory cells are characterised by an undifferentiated phenotype (Nickoloff *et al.*, 1988). In an earlier study of the WHCO-3 cell line, I have shown that the cytoskeletons of migratory cells are less differentiated than those of their non-migratory counterparts. The cytoskeletal profiles of the migratory cells were found to exhibit enhanced actin production, a reduction in high molecular weight type 1 and 2 keratins, and an accumulation of certain lower molecular weight type 1 forms (Jones, 1989). These results were achieved by the addition of 10ng/ml of exogenous EGF. Given the homology between TGF-α and EGF, the migratory response of WHCO-3 cells to 10ng/ml of exogenous TGF-α

observed in the present study is not at all surprising.

Migration requires not only changes in cytoskeletal architecture, but also the production and secretion of enzymes that facilitate cell motility by degrading components of the extracellular matrix. In a study of human epidermal raft cultures, Turksen *et al.* (1991) have shown that TGF-α, in addition to enhancing proliferation, promotes keratinocyte migration by stimulating the production and secretion of type 1 collagenase and gelatinase. Highly germane to this study is their conclusion that, in view of the connection between cell migration and tumour metastasis, TGF-α may have an important role in SCCs and tumour invasion.

Chapter 3

The Epidermal Growth Factor Receptor

3.1 Introduction

3.1.1 Receptor structure and function

The EGF-R is a 170kd protein composed of a single glycosylated polypeptide chain of 1186 amino acid residues (Carpenter and Cohen, 1990). The receptor belongs to a group of structurally and functionally related cell surface proteins that possesses an intrinsic tyrosine kinase activity (Canals, 1992). As noted in Kearsley *et al.* (1991), the EGF-R is markedly homologous to the transforming protein of the avian erythroblastosis v-*erb*B oncogene and to that of c-*erb*B2, the human equivalent of a rat neuroblastoma oncogene product designated neu. According to Nair *et al.* (1992), the receptor consists of four functional domains: an extracellular ligand-binding domain, a short transmembrane domain, a tyrosine kinase domain, and a carboxyl terminal domain (see Figure 3.1).

3.1.1.1 The extracellular ligand-binding domain

This domain comprises four subdomains and is characterised by its ability to bind to six structurally related ligands: namely, EGF, TGF-α, *vaccinia* virus growth factor, *myxoma* virus growth factor, Shope fibroma virus growth factor, and amphiregulin (Kore *et al.*, 1991). Subdomain III, which is flanked by the cystine-rich subdomains II and IV, contains major structural determinants that are important in ligand binding

(Ullrich and Schlessinger, 1990; Avivi *et al.*, 1991). Ligand-binding stimulates the receptor's tyrosine kinase domain, resulting in the phosphorylation of the receptor itself and a number of cytoplasmic substrates germane to the receptor's signal transduction mechanism (Decker *et al.*, 1992). These substrates, which include PLC-γ_1, the GTPase activating protein of *ras* (GAP) and *raf*-1 kinase, are described in Carpenter and Cohen (1990), together with a putative signalling mechanism.

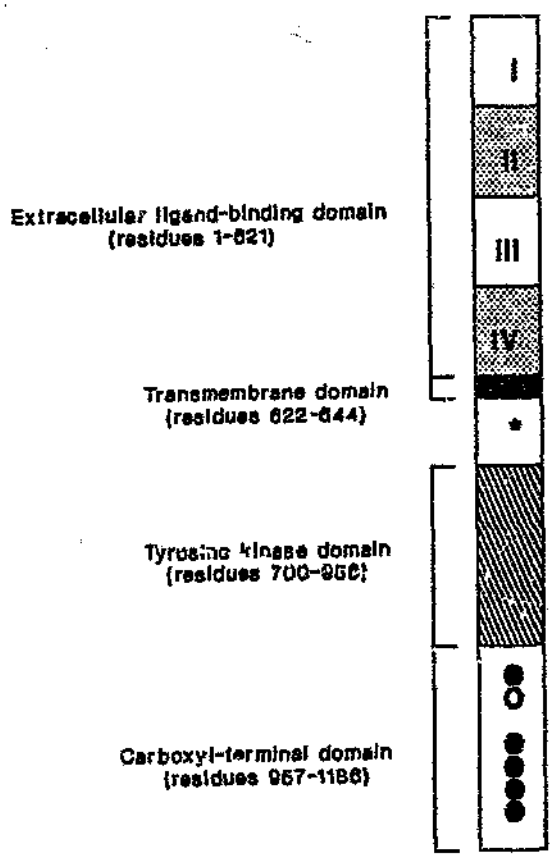


Figure 3.1 Salient features of the EGF-R (adapted from Carpenter and Cohen, 1990; and Ullrich and Schlessinger, 1990). Dotted boxes represent cystine-rich subdomains of the extracellular ligand-binding domain; asterisk and open circle represent threonine residue 654 and serine residue 1046/7 respectively; filled circles represent sites of tyrosine autophosphorylation.

Although the extracellular ligand-binding domain is generally considered to be indispensable for receptor dimerisation and hence for tyrosine kinase activity (Ullrich and Schlessinger, 1990; Canals, 1992), a study by Kwatra *et al.* (1992) has shown that EGF-Rs that lack their extracellular ligand-binding domains are still capable of dimerising.

3.1.1.2 The transmembrane domain

It is generally accepted that this alpha-helical domain does not directly influence signal transduction but is instead a passive anchor of the receptor to the membrane (Aaronson, 1991). However, a point mutation within the transmembrane domain of neu enhances its transforming properties (Ullrich and Schlessinger, 1990). Moreover, as noted in Qian *et al.* (1992), it may be the transmembrane domain that mediates EGF-R dimerisation.

3.1.1.3 The tyrosine kinase domain

This highly conserved domain comprises an ATP-binding site, an enzyme catalytic site and several regulatory sites (Ullrich and Schlessinger, 1990). The way in which ligand-binding to the extracellular domain activates the domain's catalytic site is not yet fully understood (Sorkin and Carpenter, 1991). As noted in Canals (1992), two different explanatory models have been proposed and are still in contention. According to the intramolecular model of Koland and Cerione (1988), ligand-binding induces an intramolecular conformational change which is propagated across the transmembrane domain and results in direct activation of the tyrosine kinase activity. The alternative, intermolecular model of Schlessinger (1988) proposes that ligand-binding shifts a hypothetical equilibrium between inactive monomeric receptors and active dimeric forms towards the latter because of the higher affinity of

the ligand for the dimer. Activation of the tyrosine kinase results from interactions between subunits within the receptor dimer. Despite the arguments in favour of the intramolecular model in Carraway and Cerione (1991), the balance of experimental evidence seems to favour the intermolecular model (Canals, 1992).

The phosphoryl transfer activity of the catalytic site results in phosphorylation of the receptor itself, a process termed autophosphorylation. It also causes the phosphorylation of a number of specific cytoplasmic substrates, including PLC-γ_1 (Meisenhelder *et al.*, 1989; Rotin *et al.*, 1992), GAP (Liu and Pawson, 1991), *raf*-1 kinase (Baccarini *et al.*, 1991), and many cytoskeletal components (van Bergen en Henegouwen *et al.*, 1992). Tyrosine phosphorylation modifies the function of these proteins and thereby initiates several biochemical events that ultimately lead to DNA replication and cell division (Magni *et al.*, 1991). According to Mooibroek *et al.* (1992), the activity of the catalytic site is also required for the process of ligand-induced receptor endocytosis.

3.1.1.4 The carboxyl terminal domain

Five residues for tyrosine phosphorylation are located within this domain (Vega *et al.*, 1992). In their non-phosphorylated state, these residues define an autoinhibitory region (Walton *et al.*, 1990; Sorkin *et al.*, 1991) that competes with receptor substrates for the tyrosine phosphorylating activity of the tyrosine kinase domain (Helin and Beguinot, 1991). According to Countaway *et al.* (1992), phosphorylation of serine residue 1046/7 acts as a negative regulator of kinase activity. There is evidence for a close association between the deletion of this serine residue and increased sarcomagenic potential of the v-*erb*B oncogene product. The tyrosine phosphorylated carboxyl terminal

domain determines the kinase's substrate specificity by preferentially binding to the conserved non-catalytic *src* homology domains of receptor substrates such as PLC-γ_1 and GAP (Rotin *et al.*, 1992; Vega *et al.*, 1992). The carboxyl terminal domain also plays an important role in the processes of receptor endocytosis and degradation (Decker *et al.*, 1992; Wedegaertner and Gill, 1992). Recent evidence suggests that this domain may also be important in the process of receptor dimerisation (Kwiatkowski *et al.*, 1992).

3.1.2 Desensitisation of kinase activity

Desensitisation of the EGF-R's tyrosine kinase activity provides an important physiological control over the receptor's signal transduction (Countaway *et al.*, 1992). Receptor desensitisation may be achieved in one of two ways: by signal attenuation following receptor internalisation and degradation (Cadena and Gill, 1992); or by phosphorylation of negatively regulatory receptor threonine and serine residues by non-receptor kinases (Countaway *et al.*, 1992). In the latter, the non-receptor kinases are indirectly stimulated by ligand-binding to the EGF-R and, in a positive feedback loop, utilise the receptor as one of their substrates (Carpenter and Cohen, 1990). Examples in which receptor desensitisation is mediated by these kinases include phosphorylation of threonine residue 654 by PKC and of serine residue 1046/7 by CAM II kinase (Countaway *et al.*, 1992). These enzymes do not act by reducing EGF-R dimerisation but may directly alter the receptor's conformation or indirectly alter the receptor's interaction with its cytoplasmic substrates.

3.1.3 Two classes of receptor exist

Although the EGF-R is a single gene product (Gladhaug *et al.*, 1992) that exhibits no heterogeneity in primary structure (van Belzen *et al.*, 1991), two distinct classes have been identified: a major class of low-affinity receptors and a minor class of high-affinity receptors (Berkers *et al.*, 1992).

At present there is no coherent and generally accepted explanation for this observed difference in ligand-binding affinity. According to the proponents of the intermolecular model of tyrosine kinase activation, high-affinity receptors exist as dimers that arise following ligand-induced association of low-affinity monomeric forms (Carraway and Cerione, 1991). This theory is not absolutely valid, however, because there is evidence for the existence of high-affinity monomers (De Fize *et al.*, 1989). Other researchers therefore favour the proposal that attainment of the high-affinity state is related to ligand-induced association of EGF-Rs with cytoskeletal elements (van Belzen *et al.*, 1991; van Bergen en Henegouwen *et al.*, 1992). Neither theory is consistent with the fact that high-affinity receptors must theoretically exist prior to ligand-binding (Bellot *et al.*, 1990). The recent demonstration that EGF-Rs are capable of dimerising in the absence of ligand-binding (Kwatra *et al.*, 1992) lends partial support to the view that receptor dimerisation may generate the high-affinity state, but fails to explain the existence of high-affinity monomers.

The functional significance of the two classes of EGF-R is also unclear at present. Activation of the high-affinity class may be the primary means by which EGF stimulates cells (De Fize *et al.*, 1989), although a role for the low-affinity class in EGF's signal transduction cannot be

excluded (Bellot *et al.*, 1990; Gladhaug *et al.*, 1992). Of particular relevance to the present study is the suggestion that TGF-α may be primarily dependent on the low-affinity receptor class for eliciting its mitogenic effect (Cutry *et al.*, 1988).

3.1.4 The EGF-R is often overexpressed in neoplastic cells

According to Cadena and Gill (1992), the most frequent abnormality observed in human neoplasms is the increased production of certain receptor tyrosine kinases. As noted in Veale and Thornley (1989), the first reports concerning EGF-R overproduction resulted from studies on the A431 epidermoid carcinoma cell line in 1977. Recent laboratory and clinical data suggest that many other cancer cell lines and primary human neoplasms exhibit unusually high levels of EGF-R, and that this abnormality is concomitant with rapid cellular proliferation and poor patient prognosis (Pollak *et al.*, 1991). Recent research by Barton *et al.* (1991) and Lemoine *et al.* (1992), for example, has strongly correlated the poor clinical prognosis associated with pancreatic adenocarcinoma with autocrine growth stimulatory mechanisms involving supernumary EGF-Rs. Overexpression of the EGF-R is widely regarded as a hallmark of SCCs (Ozanne *et al.*, 1986); and is found in, *inter alia*, those of the cervix (Maruo *et al.*, 1992), oral region (Kawamoto *et al.*, 1991) and oesophagus (Ozawa *et al.*, 1987; Reiss *et al.*, 1991). In the latter, such overexpression serves as a significant prognostic indicator (Ozawa *et al.*, 1989).

A substantial increase in the number of EGF-Rs may serve to promote abnormal cellular proliferation in various ways. According to Gerwin (1992), receptor overexpression may increase the probability of receptor

oligomerisation, or it may allow a cell to escape interference from negative signalling pathways. In addition, the presence of large numbers of EGF-Rs may amplify the mitogenic effect of exogenous ligand (Theodorescu *et al.*, 1991) and provide hypersensitivity to low concentrations of mitogen (Pollak *et al.*, 1991). Furthermore, the production of supernumary receptors may reduce the ability of receptor endocytosis and degradation to attenuate the mitogenic signal (Masui *et al.*, 1991; Cadena and Gill, 1992).

3.1.5 Aim of Chapter 3

As described in detail in Chapter 2, the WHCO-5 cell line secretes relatively high levels of TGF-α under unstimulated serum-free conditions. It has been proposed that this cell line's unusually short doubling time may be partly the result of a signalling defect involving overexpression of TGF-α and low-affinity EGF-Rs (Thornley and Jones, 1992). Accordingly, the aim of the experiments reported on in this Chapter is to determine the number and affinity of EGF-Rs in WHCO-5 cells by means of the ^{125}I-EGF competitive binding assay of Veale and Thornley (1989). The WHCO-3 cell line, which has been shown in this assay to produce supernumary low-affinity EGF-Rs, was used to control for experimental methodology. The well-studied A431 cell line was used for positive control purposes.

3.2 Materials and Methods

3.2.1 ^{125}I-EGF competitive binding assays

^{125}I-EGF competitive binding assays were performed according to the method described in Veale and Thornley (1989) with minor

modifications. Approximately $1.5x10^5$ cells per well were plated out in DMEM/F12 plus 5% FBS into 24 well tissue culture plates (Nunc). After 24 hours the cells were washed twice with 0.5ml/well of binding medium (DMEM/F12 plus 0.1% bovine serum albumin (BDH Chemicals)) and incubated at 37°C in a humid atmosphere of 5% CO_2 and 95% air for 30 minutes. The cells were then re-incubated in fresh binding medium for 45 minutes at 4°C. After aspiration of the medium, approximately 300 µl/well of fresh binding medium, containing 0.84 - 0.89 ng/ml of ^{125}I-human EGF (specific activity of 180.98 µCi/ g, Amersham) plus 0 - 21.86 ng/ml of unlabelled receptor grade EGF (Sigma), was added in triplicate to the wells. Non-specific binding was measured in the presence of a 1000-fold excess of unlabelled receptor grade EGF; the values appear in Appendix B. After incubation at 4°C for 3 hours, the cell monolayers were washed four times with ice-cold binding medium and solubilised in 0.5ml/well 1M NaOH for 1 hour at 37°C. ^{125}I-EGF binding assays were conducted at 4°C for a period of 3 hours because steady state binding of EGF is known to be attained in the subject cell lines under these conditions (Massagué, 1983; Veale and Thornley, 1989). Finally, the solubilised cells were transferred to 12x75-mm plastic tubes and the radioactivity was determined using a Packard Cobra auto-gamma counter. The number of cells per well was ascertained haemocytometrically by trypan blue exclusion.

3.2.2 Scatchard analysis

EGF-R number and affinity were determined from the ^{125}I-EGF competitive binding data by Scatchard analysis, using the LIGAND computer programme of Munson and Rodbard (1980). This programme is an iterative procedure for the best fit of two independent binding sites (Wang *et al.*, 1992) and, according to Veale and Thornley (1989), is

based on a statistically valid curve-fitting algorithm.

3.3 Results and Discussion

3.3.1 EGF-R expression in the A431, WHCO-3 and -5 cell lines

A431 cells are known to express an unusually high number of EGF-Rs on their cell surfaces (Ihara *et al.*, 1991). As noted in section 4.1.4 of Chapter 4, this overproduction is the result of an amplification of the receptor gene sequence. In some studies both high- and low-affinity receptor classes have been identified (De Fize *et al.*, 1989), but other studies have failed to detect the high-affinity class, and report only the presence of supernumary low-affinity receptors (Chen *et al.*, 1991). The reason for this discrepancy has not been addressed in the literature but it may be ascribable to the use of phenotypically different sublines. The WHCO-3 cell line is known to produce exceptionally high numbers of low-affinity EGF-Rs. This may be a result of direct xenotransplantation from the original tumour biopsy (Veale and Thornley, 1989) but, as discussed in Chapter 4, may well be attributable to EGF-R gene amplification.

The ^{125}I-EGF competitive binding data obtained in the present study for the A431, WHCO-3 and -5 cell lines is summarised in Appendix B (see Tables B1, B2 and B3 respectively). Scatchard analysis of this data produced linear plots that are typical of those obtained in studies of systems comprising single affinity ligand-binding sites (see Figure 3.2).

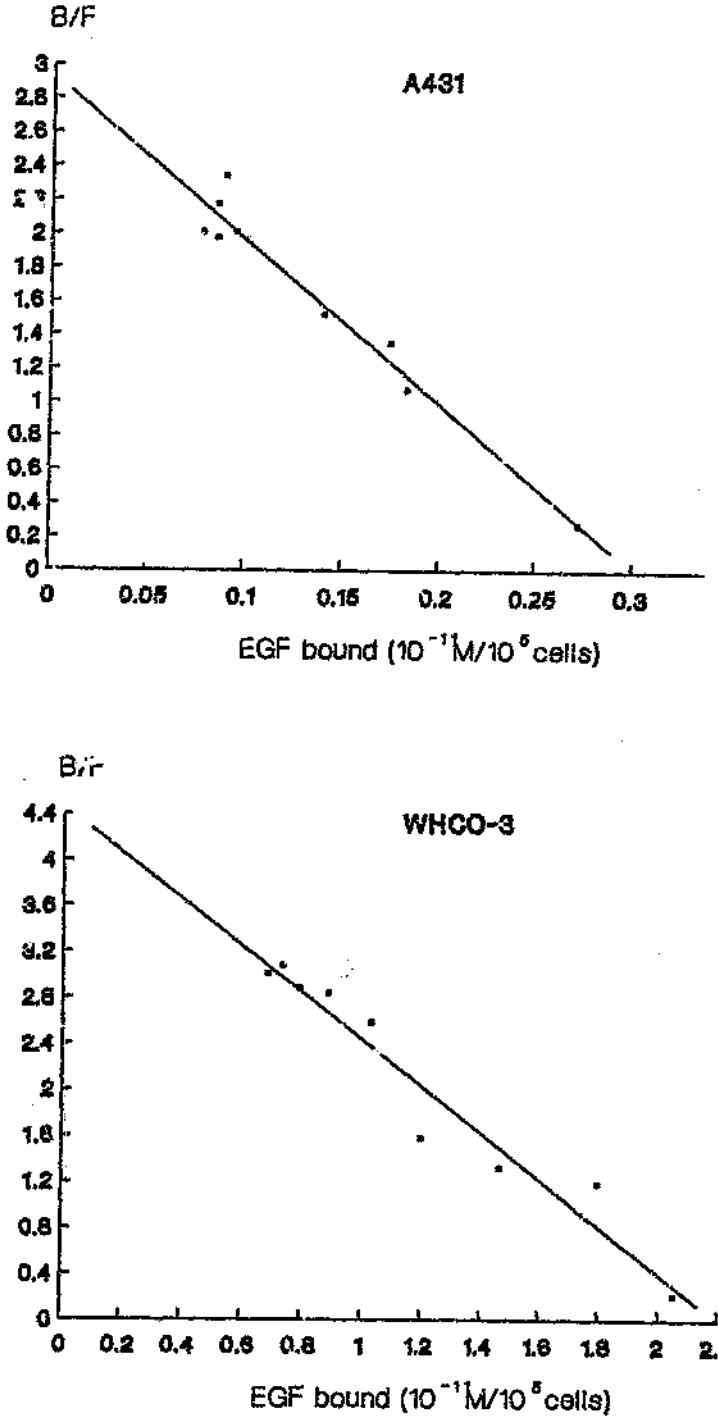


Figure 3.2 Scatchard analysis of ^{125}I-EGF binding for the A431 and WHCO-3 cell lines. The ^{125}I-EGF binding assays were performed at 4°C as described in section 3.2.1. Each data point represents the average of triplicate determinations from a single experiment. The *ordinate* indicates the ratio of bound to free (B/F) ^{125}I-EGF and the *abscissa* indicates the molar concentration of bound EGF per 10^5 cells.

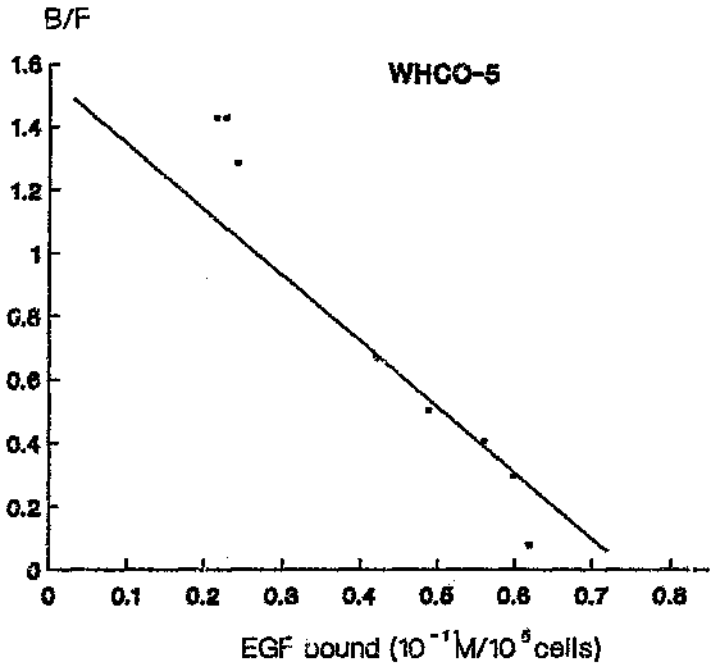


Figure 3.2 (continued) Scatchard analysis of ^{125}I-EGF binding for the WHCO-5 cell line. The ^{125}I-EGF binding assays were performed at 4°C as described in section 3.2.1. Each data point represents the average of triplicate determinations from a single experiment. The *ordinate* indicates the ratio of bound to free (B/F) ^{125}I-EGF and the *abscissa* indicates the molar concentration of bound EGF per 10^5 cells.

Calculation of the number of receptors per cell and the receptors' dissociation constants for the A431 and WHCO-3 cell lines produced values that are in good agreement with those reported in the literature (see Table VI).

Although the dissociation constant calculated for the WHCO-3 cell line is somewhat smaller than that reported previously, its magnitude is compatible with those given for EGF-Rs that are considered to belong to the low-affinity receptor class (van Belzen *et al.*, 1991; Gladhaug *et al.*, 1992; Hongo *et al.*, 1992). Scatchard analysis of the ^{125}I-EGF competitive

binding data for the WHCO-5 cells showed that they express $6.4x10^5$ low-affinity EGF-Rs per cell, a figure which is approximately 12 times higher than the corresponding figure for normal oesophageal keratinocytes (see Table VI).

Table VI Number of EGF receptors per cell and dissociation constants

Cell line	Dissociation constant (K_d)	Number of receptors per cell	Reference
Normal oesophageal keratinocytes	$4.2x10^{-9}$M	$5.5x10^5$	Veale and Thornley, 1989
A431	$1.4x10^{-9}$M $1.1x10^{-9}$M	$2.8x10^5$ $2.5x10^6$	present study Chen et al., 1991
WHCO-1	$2.2x10^{-9}$M	$4.2x10^5$	Veale and Thornley, 1989
WHCO-3	$6.2x10^{-10}$M $1.1x10^{-9}$M	$12.9x10^5$ $10.4x10^5$	present study Veale and Thornley, 1989
WHCO-5	$4.2x10^{-10}$M	$6.4x10^5$	present study

Each analysis in the present study consisted of 8 to 9 different unlabelled EGF concentrations performed in triplicate. The number of receptors per cell and dissociation constants were calculated from ^{125}I-EGF binding data using the LIGAND computer programme of Munson and Rodbard (1980). Cell numbers were determined haemocytometrically by trypan blue exclusion.

As discussed in Chapter 2, the WHCO-5 cell line secretes relatively high levels of TGF-α under unstimulated serum-free conditions. It has been proposed that the rapid *in vitro* growth characteristics of this cell line may partly be the result of an autocrine signalling defect involving overexpression of TGF-α and low-affinity EGF-Rs (Thornley and Jones, 1992). The present demonstration of supernumary low-affinity EGF-Rs on the surface of these cells lends experimental support to this proposal.

3.3.2 EGF-R overexpression and neoplastic transformation

There is persuasive experimental evidence to support a positive role for EGF-R overexpression in the processes of cellular transformation *in vitro* and tumourigenesis *in vivo*. Overexpression of EGF-Rs in NIH 3T3 fibroblasts following transfection with a human EGF-R cDNA expression vector results in ligand-dependent transformation *in vitro* (Di Fiore *et al.*, 1987; Riedel *et al.*, 1988). Recent work by Masui *et al.* (1991) has shown that lipofection of NR6 3T3-derived cells with a wild-type EGF-R expression vector produces cells that are slightly tumourigenic in athymic mice; and that lipofection of NR6 cells with a mutant EGF-R sequence encoding receptors that are incapable of being down-regulated results in significantly enhanced tumourigenicity *in vivo*.

Neoplastic transformation *in vivo* is a multi-step process: firstly, the normally interdependent systems controlling proliferation and differentiation must be uncoupled; secondly, proliferation must be stimulated in such a way as to result in extensive division of the transformed cells (Cross and Dexter, 1991). It is unclear whether EGF-R overexpression has a primary causative role in this process or merely acts to maintain or enhance it. In the aforementioned studies of Di Fiore *et al.* (1987) and Riedel *et al.* (1988), it is claimed that such overexpression, coupled with the unmasking effects of ligand binding, is a sufficient cause of transformation in NIH 3T3 mouse fibroblasts. It must be noted, however, that their *in vitro* studies focused on cells that were already immortalised; and that, as regards *in vivo* systems, the authors considered their findings to be suggestive rather than conclusive. According to Pollak *et al.* (1991), the expression of excessive numbers of EGF-Rs is more likely to contribute to the progression of

neoplastic transformation rather than to cause its initiation. Extensive clinical evidence strongly supports the role of excessive EGF-R signalling in the progression of many types of human cancers (Rademann *et al.*, 1992). Autocrine activation of overexpressed EGF-Rs may promote progression by conferring a selective growth advantage on an already aberrant preneoplastic cell (Hollstein *et al.*, 1988). The role of the EGF-R in maintaining the tumourigenic properties of cancer cells is well illustrated by the ability of specific neutralising antibodies that bind to the EGF-R to reduce the proliferation of SCCs *in vitro* (Reiss *et al.*, 1991) and to suppress the tumourigenicity of EGF-R-transfected NR6 cells *in vivo* (Masui *et al.*, 1991).

3.3.3 The EGF-R and oesophageal cancer

For the purposes of the present study, it should be noted that the question whether EGF-R overexpression, coupled with ligand-binding, has a primary or a secondary role in carcinogenesis is not of the essence. What is important is the undeniable fact that it is clearly implicated, at least by virtue of the growth advantage it confers, in the multi-step process of neoplasia.

A link between neoplasia and the hyperproduction of EGF-Rs has been demonstrated in many different cell types since it was first reported in connection with A431 cells in 1977. Such overexpression has been described as the hallmark of SCCs (Ozanne *et al.*, 1986). Regarding human oesophageal SCCs, although there are examples characterised by a relatively low number of high-affinity receptors (Banks-Schlegel and Quintero, 1986), there is considerable evidence to support a positive correlation between this type of cancer and the production of supernumary low-affinity EGF-Rs. Such overexpression has recently

been noted in a number of established oesophageal SCC cell lines (Veale and Thornley, 1989; Yoshida *et al.*, 1990; Chen *et al.*, 1991). The results of the present study in respect of the WHCO-3 and -5 cell lines confirm and extend those reported in Veale and Thornley (1989). A functional role for overexpressed EGF-Rs *in vivo* is clearly suggested by the demonstration of excessive levels of EGF-R mRNA and protein in several primary oesophageal SCCs (Ozawa *et al.*, 1987; Hollstein *et al.*, 1988).

From Table VI it may be deduced that the WHCO-1, -3 and -5 cell lines produce between 8 and 23 times as many low-affinity cell surface EGF-Rs as do normal oesophageal keratinocytes. As shown in Chapter 2, these cell lines secrete significant quantities of TGF-α under unstimulated serum-free conditions. Hyperproduction of low-affinity EGF-Rs is significant in view of the fact that TGF-α may be primarily dependent on this class of EGF-R for eliciting its mitogenic signal (Cutry *et al.*, 1988). Taken together, my results support the speculation, which will be elaborated in Chapter 5, that TGF-α and the EGF-R act in an autocrine mode to regulate the *in vitro* growth behaviour of these cell lines.

As mentioned in section 3.1.4, overexpression enhances abnormal proliferation in various ways. One such way is the promotion of receptor oligomerisation. A second is the reduction of interference by negative signalling pathways that would otherwise desensitise the EGF-R tyrosine kinase activity by phosphorylating negative regulatory residues in the receptor's intracellular region. Thirdly, the presence of excess receptors may provide hypersensitivity to mitogenic stimulation. Finally, receptor hyperproduction may result in the reduced ability of receptor endocytosis and degradation to attenuate the mitogenic signal.

There remains for consideration the important question of what causes

EGF-R hyperproduction. This forms part of the subject- matter of the next Chapter.

Chapter 4

Gene Amplification

4.1 Introduction

4.1.1 Cancer and genetic instability

There is compelling evidence that cancer is a process that involves multiple genetic lesions (Hunter, 1991). These lesions, whether initiating or progression-associated events, may be mediated through gross chromosomal changes and thus be cytogenetically visible. The common tumour chromosome aberrations fall into two broad classes: structural and numerical. The former includes translocations, inversions, deletions and amplifications; the latter comprises losses or duplications of entire chromosomes (Solomon *et al*., 1991).

As discussed in section 1.2.2 of Chapter 1, two classes of genes are implicated in cancer. First, there are the proto-oncogenes, which can be converted from normal cellular genes to oncogenes by a variety of submicroscopic events including point mutations, small insertions and deletions, and juxtaposition to other chromosome sequences. Juxtaposition can be visualised cytogenetically as a translocation or inversion. At least three oncogenes - *myc*, *rel* and *abl* - are known to result from structural rearrangement of proto-oncogenes. The second type comprises the tumour suppressor genes, which, in contrast to oncogenes, contribute to oncogenicity through their loss rather than their activation. Their behaviour is recessive, and both copies must be inactivated for tumour formation to occur. In general, structural

rearrangements that consistently juxtapose two different chromosomal regions are considered to contain dominant oncogenic sequences; deletions or monosomies are thought to be the site of recessive tumour suppressor genes (Solomon *et al.*, 1991).

Oncogenes are often found to be amplified in advanced tumours, presumably because their overexpression through amplification confers a selective growth advantage (Stark *et al.*, 1989). The present Chapter is concerned with a possible role for amplification of the TGF-α and EGF-R genes in the cell lines that form the basis of this study.

4.1.2 Manifestations and stages of gene amplification

Since its discovery in drug-resistant eukaryotic cells, somatic amplification of specific genes has been implicated in an increasing variety of the adaptive responses of cells to environmental stresses (Alitalo and Schwab, 1986).

According to Stark *et al.* (1989), amplified DNA can often be observed by light microscopy either as an extended chromosomal region (ECR) or as extrachromosomal elements called double minute chromosomes (DMs). Extrachromosomal elements play an important role in amplification, and it now seems likely that their formation is coupled to chromosomal deletions and perhaps to other chromosomal abnormalities such as inversion, translocation and possibly even chromosome loss.

Amplifications are selected *in vitro* in distinct steps of increasing drug concentration (Stark *et al.*, 1989). The primary event of amplification takes place in a single cell and thus has not been amenable to direct

study so far. Secondary events, not necessarily selected for by the drug, may then follow during growth to a clonal population sufficiently large for analysis. If the amplified DNA is extrachromosomal in this first-step clonal population, increasing the drug concentration in a second step will simply select cells with more of the same element. If the amplified DNA is intrachromosomal, however, a second and distinct amplification event, again taking place in a single cell, is required to generate the additional copies of the target gene for survival.

As noted by Stark *et al.* (1989), it is unclear whether DMs and ECRs represent different outcomes of the same primary event or arise from fundamentally different processes. DMs can integrate to generate ECRs at random chromosomal locations, whereas ECRs are sometimes found at the native locus. During amplification, DNA sequences from different parts of the genome are joined together to form novel structures. Most of the amplified DNA is represented by a single rearranged structure that is formed at an early stage of the process and subsequently reamplified. The preferential nature of the process suggests that the rearrangement of DNA creates or brings close to the selected gene certain sequences that act in *cis* to favour amplification.

4.1.3 Mechanisms of gene amplification

As noted by Alitalo and Schwab (1986), there is evidence that illegitimate DNA replication occurs to a limited extent in normal cells. In unselective conditions this DNA is probably lost, perhaps via the formation of micronuclei. However, if there is selective pressure to retain an increased gene dosage then a progressive multiplication of gene copy number ensues.

The incidence of cells bearing amplified genes under conditions of cytotoxic selection can vary by two orders of magnitude and is greatly increased by the presence of mitogenic substances. But what causes amplification? One putative mechanism is provided by the so called "onion-skin" model depicted in Figure 4.1.

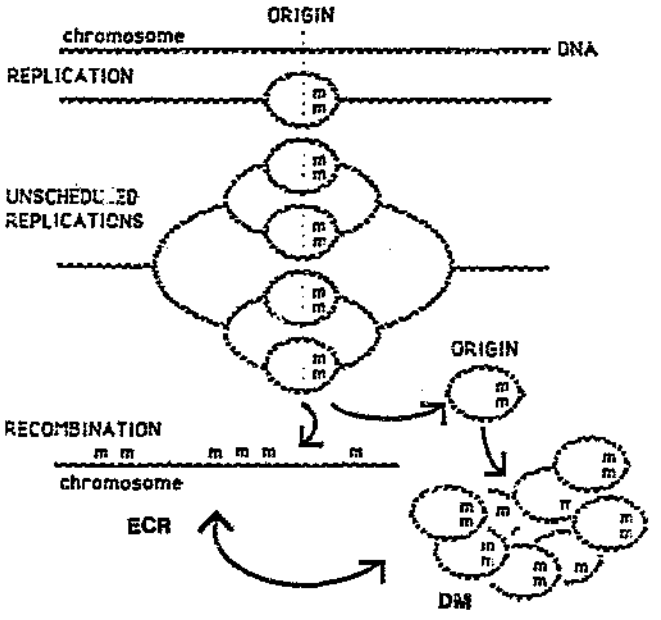


Figure 4.1 "Onion-skin" model of DNA amplification in tumour cells. The m denotes a hypothetical (onco)gene within the amplified domain. As explained in section 4.1.3, unscheduled replication of DNA results in multicopies that must be resolved during the cell cycle into DMs or ECRs.

The following explanation of the model is drawn from Alitalo and Schwab (1986). In the top part of the Figure, chromosomal DNA is replicated bidirectionally from a fixed site of origin during the S phase of the cell cycle. With a certain probability, unscheduled replications of the already replicated DNA may occur during a single cell cycle, leading to a structure shown as superimposed replication bubbles. The multiple

copies of DNA must resolve into a linear array before the next mitosis. This can occur by homologous or illegitimate recombinations between different amplified segments. The process can lead to a linear, tandem but heterogenous multicopy unit, shown on the left, which may in some cases evolve into an ECR. If suitable recombinations occur, extrachromosomal circular DNA elements containing origins for DNA replication are formed, which may be the precursors of DMs. The m denotes a hypothetical (onco)gene within the amplified domain.

It should be noted, however, that no clear-cut cause of amplification has been established and that several rival theories still compete to explain its mechanisms (Bishop, 1991). These theories fall into two classes: in the one, over-replication within a single cell cycle is responsible for generating the extra copies of DNA; in the other, unequal segregation is involved and the control of replication within each cell cycle is normal. Five possible models are described in Stark *et al.* (1989).

4.1.4 Tumour specificity of oncogene amplification

The transition of proto-oncogenes to oncogenes through some form of genetic damage seems to have a specific or patterned association with different kinds of human malignancies, as shown in Table VII from Bishop (1991).

EGF-R gene amplification has been found in several tumours, including gliomas and SCCs (Wood *et al.*, 1992). By far the most commonly amplified gene in gliomas, particularly glioblastomas, is that encodig the EGF-R (Torp *et al.*, 1991; Fuller and Bigner, 1992). The EGF-R gene is rearranged in a significant percentage of gliomas in which it is amplified, resulting in extracellular domain deletions that yield

truncated EGF-Rs (Humphrey *et al.*, 1991). Furuta *et al.* (1992) also observe that overexpression of the EGF-R in certain other human tumours, particularly SCCs, is the result of receptor gene amplification.

It may be necessary, however, to distinguish between results obtained from primary tumours and those from cell lines derived from such tumours. Thus, Yamamoto *et al.* (1986) found EGF-R gene amplification in only one out of six primary SCC tumours but in ten out of twelve SCC cell lines, of which three out of five were oesophageal. The incidence of amplification has also been found to be relatively low in primary SCCs of the head and neck (El-Zayat *et al.*, 1991; Kearsley *et al.*, 1991), the nasal cavity and paranasal sinuses (Furuta *et al.*, 1992), and the oesophagus (Hollstein *et al.*, 1988).

Of particular relevance to the present study is the research that has been conducted on the A431 cell line and several oesophageal SCCs. Overexpression of the EGF-R in A431 cells is principally the result of receptor gene amplification and mRNA hyperproduction (Lin *et al.*, 1984). In these cells, the receptor gene is also known to be translocated and rearranged (Ullrich *et al.*, 1984). As regards oesophageal SCCs, amplification of the EGF-R gene has been reported both in tumour-derived cell lines (Yamamoto *et al.*, 1986; Yoshida *et al.*, 1990) and in primary tumour samples (Lu *et al.*, 1988; Hollstein *et al.*, 1988), although the frequency of amplification in the latter is relatively low (Hollstein *et al.*, 1988). On the other hand, amplification of the TGF-α gene has not been detected in oesophageal SCC cell lines (Yoshida *et al.*, 1990) and appears to be a rare event, the only reference known to me being Barrett-Lee *et al.* (1990) concerning an isolated mammary dysplasia.

Table VII Proto-oncogenes and human tumours: some consistent associations

Proto-oncogene	Neoplasm(s)	Lesion
abl	Chronic myelogenous leukemia	translocation
EGF-R (*erb*B-1)	Squamous cell carcinoma; astrocytoma	amplification
***neu* (*erb*B-2)**	Adenocarcinoma of breast, ovary and stomach	amplification
gip	Carcinoma of ovary and adrenal gland	point mutations
gsp	Adenoma of pitiutary gland; Carcinoma of thyroid	point mutations
myc	Burkitt's lymphoma Carcinoma of lung, breast and cervix	translocation amplification
L-*myc*	Carcinoma of lung	amplification
N-*myc*	Neuroblastoma; Small cell lung carcinoma	amplification
H-*ras*	Carcinoma of colon, lung and pancreas; Melanoma	point mutations
K-*ras*	Acute myelogenous and lymphoblastic leukemia; Carcinoma of thyroid; Melanoma	point mutations
N-*ras*	Carcinoma of genitourinary tract and thyroid; Melanoma	point mutations
ret	Carcinoma of the thyroid	rearrangement
ros	Astrocytoma	?
K-*sam*	Carcinoma of stomach	amplification
sis	Astrocytoma	?
src	Carcinoma of colon	?
trk	Carcinoma of thyroid	rearrangement

4.1.5 Role of oncogene amplification in carcinogenesis

As noted by Alitalo and Schwab (1986), amplification of certain oncogenes is a common correlate of the progression of some tumours but also occurs as a rare sporadic event in various oncogenes in different types of tumour. Amplified oncogenes, whether tumour-type specific or

sporadic, are also expressed at elevated levels, sometimes in cells in which their diploid forms are silent. It is clear, therefore, that increased expression of an amplified oncogene may contribute to the multi-step progression of at least some cancers (Velu, 1990; Gullick, 1991), including those of the brain (Torp *et al.*, 1991) and, which is of particular interest in the context of the present study, SCCs of the oesophagus (Hollstein *et al.*, 1988).

This conclusion is reinforced by Bishop (1991), who notes that gene amplification is one of four kinds of predispositions to genetic change that are associated with progression, the others being the loss or gain of entire chromosomes, widespread loss of intrachromosomal domains, and heightened susceptibility to spontaneous and induced mutations. As he observes, there may be no requisite order of events in progression, which appears to be a phenomenon characterised by the accumulation rather than the sequence of genetic damage.

4.1.6 Aim of Chapter 4

In Chapter 2 it has been shown that the WHCO cell lines, especially WHCO-5, constitutively synthesise and secrete relatively high levels of TGF-α; and Chapter 3 has demonstrated that these cell lines produce supernumary low-affinity EGF-Rs. The aim of the present Chapter is to report on the results of DNA hybridisation experiments designed to determine whether these observations are associated with amplification of the TGF-α and EGF-R genes. In the quantitative assessment of the experimental results, the A431 cell line was used as a control and standard.

4.2 Materials and Methods

4.2.1 Plasmid DNA extraction

Escherichia coli transformants containing plasmid DNA bearing TGF-α and EGF-R cDNA inserts were grown overnight at 37°C in Luria-Bertani medium (see section C1 of Appendix C) plus 50μg/ml ampicillin (Boehringer Mannheim). The plasmid DNA was bulk-extracted according to the alkali-lysis method of Sambrook *et al.* (1989; see section C2 of Appendix C) and stored at -20°C until required.

4.2.2 Genomic DNA extraction

The extraction of genomic DNA from the subject cell lines was based on the salt method of Miller *et al.* (1988) and is described in section C3 of Appendix C. The concentration of genomic DNA was determined at 260nm using a Beckmann Du-64 spectrophotometer (see section C4 of Appendix C).

4.2.3 Gene probe preparation

The three DNA probes used in the present study were: the 1.3kb full-length TGF-α cDNA insert from plasmid pS65C17N13 (Derynck *et al.*, 1984); the linearised 7.0kb EGF-R plasmid (American Type Culture Collection) encoding a 2.4kb EGF-R internal cDNA sequence (Xu *et al.*, 1984); and the 0.9kb β-globin intervening sequence II from plasmid vector pBR322.

To excise and isolate the TGF-α and β-globin inserts from their respective plasmids, the latter were restricted and electrophoretically

separated at 100mV on 0.8% TAE agarose gels (see section C5 of Appendix C). Bacteriophage λ II markers (Boehringer Mannheim) were used for calibration purposes. The restriction fragments that corresponded in size to those expected for the two inserts were cut out of the gels and purified using a Geneclean kit (Bio 101 Inc.; see section C6 of Appendix C). The TGF-α insert was excised from plasmid pS65C17N13 using the *Eco*RI restriction endonuclease. Excision of the 0.9kb β-globin insert from plasmid vector pBR322 required a double digestion involving both the *Eco*RI and *Bam*H1 endonucleases. The EGF-R probe was prepared by linearising the entire EGF-R plasmid using the *Hind*III restriction endonuclease. The restriction enzymes were purchased from Boehringer Mannheim and used according to the manufacturer's specifications. In order to check both the accuracy of the endonuclease restriction steps and the efficiency of the DNA purification procedure, the restricted TGF-α and β-globin plasmids, together with their purified inserts and the linearised EGF-R plasmid, were electrophoretically separated at 100mV on a 0.8% TBE agarose gel (see sections C7 and C8 of Appendix C).

Prior to labelling, the concentration of the purified TGF-α insert and the linearised EGF-R plasmid was determined by comparison with known quantities of a 2.7kb *Eco*RI-linearised pUC18 plasmid vector (see section C9 of Appendix C). The concentration of the β-globin probe was determined spectrophotometrically.

The three gene probes were finally labelled to a specific activity of 0.67 to 3.07×10^9 dpm/μg DNA with deoxyadenosine 5'[α^{32}P] triphosphate (3000Ci/mmol; Amersham) using a Multiprime DNA labelling kit (Amersham; see section C10 of Appendix C). The specific activity was calculated using the equations set out in section C10.1 of Appendix C.

4.2.4 Dot-blot hybridisation

The genomic DNA samples were initially denatured at 100°C for a period of 10 minutes. DNA in the range of 0.5 to 10.0μg was then dotted under vacuum onto a nitrocellulose membrane (Hybond-C extra, Amersham) using a Bio-Dot microfiltration unit (Bio-Rad) according to the manufacturer's instructions. The DNA was fixed by baking the nitrocellulose membrane in a vacuum oven at 80°C for 2 hours. Prehybridisation of the membrane was carried out at 42°C for approximately 4 hours in prehybridisation solution (see section C11 of Appendix C). DNA hybridisation was performed by incubating the membrane in hybridisation solution (prehybridisation solution containing labelled probe) for 16 hours at 42°C. Stringency washes were carried out at 68°C for 2x30 minutes in a 0.1% SDS solution containing consecutively 2x, 1x and 0.5x standard saline citrate (SSC; see section C11.2 of Appendix C). The membrane was exposed at -70°C to Fuji Medical X-ray film for 24 to 48 hours. Prior to reprobing, the nitrocellulose was stripped of probe by agitating in boiling 0.1% SDS for 1 hour at room temperature. The autoradiographs were scanned using a Zeineh laser densitometer and the densitometric data processd by an IBM 1-D autostepover/videophoresis programme (Biomedical Instruments).

4.3 Results and Discussion

4.3.1 Results of DNA hybridisation experiments

Autoradiographs of the dot-blot DNA hybridisation analyses are shown in Figures 4.2 and 4.3. Panel a of the Figures shows genomic DNA of the subject cell lines probed with the 1.3kb *Eco*RI TGF-α cDNA insert and

with the *Hind*III-linearised EGF-R plasmid respectively. Panel b of the Figures shows the same DNA probed with the 0.9kb β-globin intervening sequence. Hybridisation with the β-globin probe was used to standardise for DNA sample loading and to act as an internal control in the quantitative assessment of gene amplification.

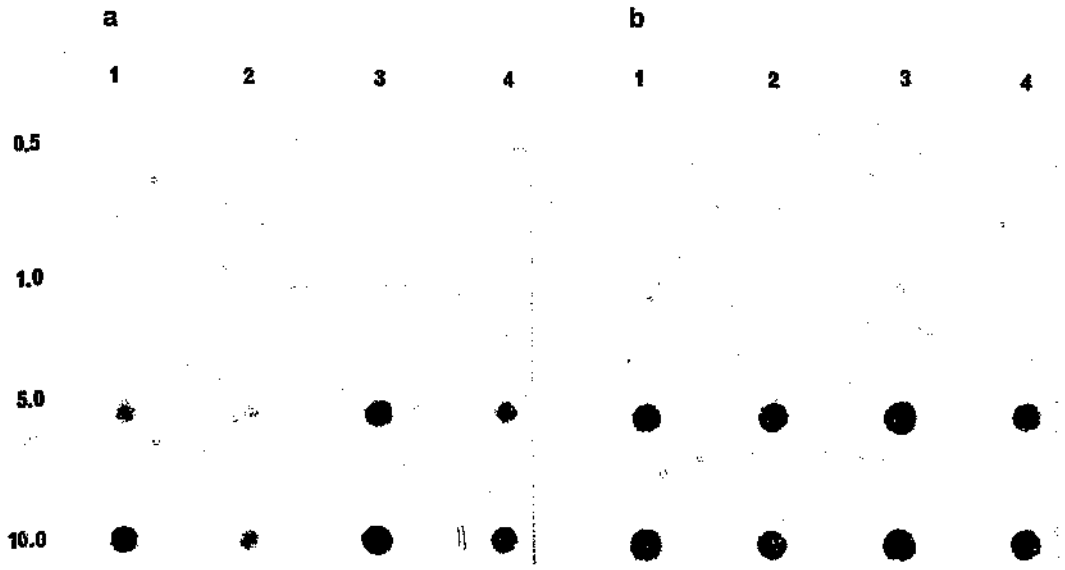


Figure 4.2 Dot-blot DNA hybridisation analysis of the TGF-α and β-globin genes. Panel a - autoradiograph showing hybridisation of genomic DNA to the 1.3kb *Eco*R1 TGF-α cDNA probe; exposure time = 2 days. Panel b - autoradiograph showing hybridisation of the same DNA to the 0.9kb β-globin intervening sequence II probe; exposure time = 2 days. The quantity of genomic DNA (μg) is indicated on the left; lanes 1 to 4 are A431, WHCO-1, -3 and -5 respectively.

The relative intensity of panel a compared with panel b in each of the two Figures is a qualitative reflection of the degree of gene amplification. No amplification is indicated in Figure 4.2 whereas Figure 4.3 exhibits significant though varying amplification for each cell line.

In order to quantify the degree of gene amplification, the intensity of the hybridisation signals emitted from the 10μg array of genomic DNA

samples was measured using a Zeineh laser densitometer; the results are shown in columns 2 to 4 of Table VIII. The figures in these columns are density-curve integrals (dci's) based on an average of at least two readings.

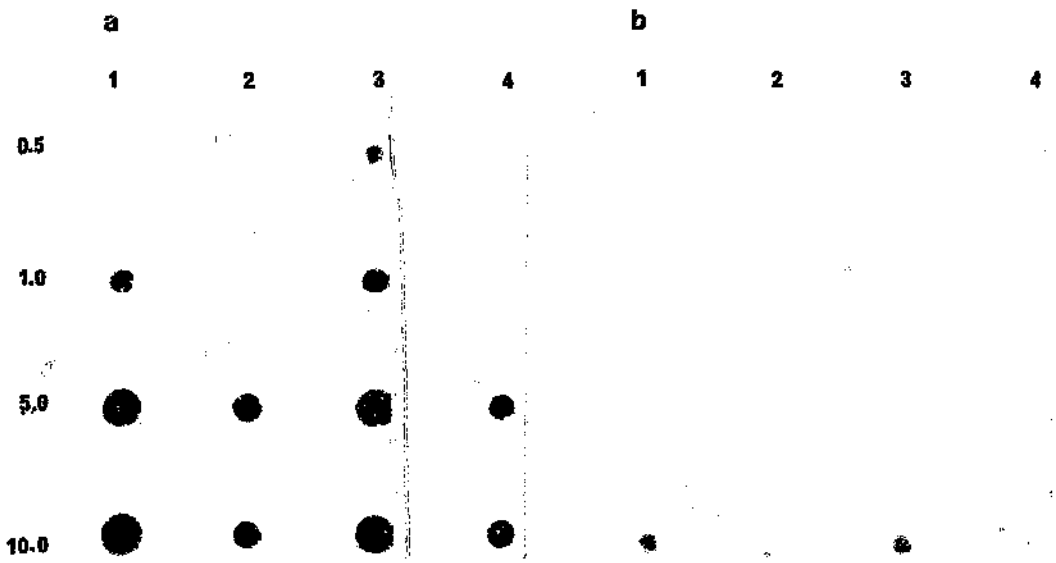


Figure 4.3 Dot-blot DNA hybridisation analysis of the EGF-R and β-globin genes. Panel **a** - autoradiograph showing hybridisation of genomic DNA to the 7.0kb linearised EGF-R plasmid; exposure time = 1 day. Panel **b** - autoradiograph showing hybridisation of the same DNA to the 0.9kb β-globin intervening sequence II probe; exposure time = 1 day. The quantity of genomic DNA (μg) is indicated on the left; lanes 1 to 4 are A431, WHCO-1, -3 and -5 respectively.

The ratio of column 2 (or column 3) to column 4 may be used to calculate gene amplification. Thus, as regards the A431 cell line, the ratio of the value in column 3 to that in column 4 (27539.0/17079.5 = 1.612) is deemed to correspond to the accepted 30-fold amplification of the EGF-R gene in this cell line (Merlino *et al.*, 1984; Gope *et al.*, 1991). Hence, amplification of the EGF-R gene in the WHCO cell lines may be calculated as their corresponding ratios divided by 1.612 (the A431 ratio) and multiplied by 30. Amplification of the TGF-α gene may be calculated

in the same way, *mutatis mutandis*, except that the multiplicative factor is not 30 but unity because, to the best of my knowledge, there is no evidence for TGF-α gene amplification in the A431 cell line. The degree of amplification for the TGF-α and EGF-R genes is shown in rounded form in columns 5 and 6 of Table VIII respectively.

Table VIII TGF-α and EGF-R gene amplification

Cell line	dci (TGF-α)	dci (EGF-R)	dci (β-globin)	Gene amplification	
				TGF- α	EGF-R
A431	15300.5	27539.0	17079.5	1x	30x
WHCO-1	8538.5	4951.0	11644.5	1x	8x
WHCO-3	22653.0	22223.7	16423.0	2x	25x
WHCO-5	17960.5	6487.5	13027.5	2x	9x

The amplification figures were calculated as described in section 4.3.1. Amplification is deemed to have been detected only if it exceeds 3-fold.

4.3.2 Amplification of the TGF-α gene

As discussed in Chapter 2, the WHCO-1, -3 and -5 cell lines secrete appreciable quantities of TGF-α under unstimulated serum-free conditions. The quantity secreted by WHCO-5 is approximately 41 times that secreted by the A431 cell line and appears to be relatively high for a neoplastic cell line. Using the A431 cell line as a negative control and standard, I have shown above that this oversecretion is not the result of TGF-α gene amplification. The WHCO-1 and -3 cell lines - which, as may be inferred from Table III, secrete respectively 1.5 and 2.5 times as much TGF-α as the A431 cell line - also lack amplified TGF-α sequences.

These results are consistent with those reported elsewhere. Yoshida *et*

al. (1990) did not detect TGF-α gene amplification or rearrangement in a number of human oesophageal SCC cell lines that expressed high levels of TGF-α mRNA. Of course, TGF-α mRNA overexpression is not a sufficient condition for oversecretion of TGF-α protein. Because cleavage is a rate-limiting step in the generation of soluble TGF-α, it would appear that an enhanced ability of cells to cleave TGF-α from their transmembrane precursors is also a necessary condition for TGF-α oversecretion. The mechanisms associated with enhanced pro-TGF-α cleavage have been discussed at length in Chapter 2.

4.3.3 Amplification of the EGF-R gene

In oesophageal SCCs, it has been demonstrated in several studies that overexpression of the EGF-R is frequently associated with receptor gene amplification. In primary oesophageal tumours, Lu *et al.* (1988) have reported at least 4-fold amplification, and Hollstein *et al.* (1988) at least 10-fold amplification. Regarding derived cell lines, unspecified levels of amplification are reported in Yamamoto *et al.* (1986) and Yoshida *et al.* (1990).

In the light of the above, the existence of amplified EGF-R gene sequences in the WHCO cell lines (see Table VIII) is unsurprising; moreover, the degree of amplification is not inconsistent with that reported elsewhere. As may be seen from Table IX, there is a strong positive correlation between EGF-R gene amplification and protein overexpression in the WHCO cell lines, which was noted in Jones *et al.* (1992).

A similar relationship has been demonstrated in clonal sublines of A431 cells (Lin *et al.*, 1984), and in certain other SCC cell lines (Reiss *et al.*,

1991). Although levels of EGF-R mRNA were not evaluated in the present study, it is no matter for surprise that there exists a positive correlation between EGF-R gene amplification and an intermediate stage in the hyperproduction of its protein, namely overexpression of EGF-R mRNA, in oesophageal SCCs (Lu *et al.*, 1988; Yoshida *et al.*, 1990; Hollstein *et al.*, 1988).

Table IX Comparison of EGF-R gene amplification and protein overexpression

Cell line	EGF-R gene amplification	Level of EGF-R protein expression[1]
WHCO-1	8x	8x
WHCO-3	25x	23x
WHCO-5	9x	12x

1 level measured relative to that of normal oesophageal keratinocytes, from Chapter 3, Table VI.

Extra gene copies can arise from a true gene amplification - that is, duplication of a chromosomal region localised around the gene - or from polyploidy of chromosome 7, which bears the EGF-R gene locus (Hurtt *et al.*, 1992). One would speculate that the higher the observed level of EGF-R gene amplification, the less likely it is that chromosome 7 polyploidy has a significant role. Thus, karyotypic studies of the WHCO-3 cell line, which has a high level of receptor gene amplification, have shown that such amplification is not the result of chromosome 7 polyploidy, but may arise from tandem gene duplications (Heiss, 1992). It is unknown whether polyploidy of chromosome 7 is implicated in EGF-R gene amplification in the WHCO-1 and -5 cell lines as no karyotypic studies of these lines have been undertaken to date.

In concluding this discussion, it is appropriate to note that the

expression of supernumary EGF-Rs may arise from mechanisms other than gene amplification, including mRNA overexpression (Hollstein *et al.*, 1988; Lemoine *et al.*, 1992), defective receptor downregulation (Masui *et al.*, 1991; Reiss *et al.*, 1991) and ligand-induced receptor production (Bjorge *et al.*, 1989; Kearsley *et al.*, 1991). Indeed, one or other of these mechanisms may play a contributory role even in the presence of gene amplification.

Chapter 5

Concluding Discussion

5.1 Aim of Chapter 5

Like Janus, this Chapter looks forward as well as backward, because an ending is also a beginning. Its chief concern is retrospective - namely, the development of a synoptic view of the main substantive points of Chapters 2, 3 and 4. This is presented in sections 5.2 to 5.5. The prospective final section puts forward some thoughts on the structuring of future research on the WHCO cell lines.

5.2 Transforming growth factor alpha (Chapter 2)

5.2.1 Constitutive production and secretion of TGF-α

The production and secretion of TGF-α by the A431 and WHCO cell lines and their biological response to exogenous mature TGF-α were examined in Chapter 2.

My results show that, under unstimulated serum-free conditions (section 2.3.1), the A431 and WHCO cell lines constitutively secrete appreciable quantities of TGF-α over a period of 20 hours. The level of TGF-α secretion by WHCO-5 cells appears to be relatively high for a neoplastic cell line and its short doubling time may be attributable to signalling defect involving constitutive overexpression of TGF-α and the EGF-R,

possibly coupled with the relative dysfunction of some inhibitory factor such as TGF-β. Two distinct species of TGF-α were found in the lysates of unstimulated cells. The lower molecular weight forms were consistent with reports in the literature for various species of pro-TGF-α. The higher molecular weight forms were likewise consistent with known findings for meso-TGF-α and are of particular interest because, being cell-associated rather than secreted, they are suggestive of an autocrine mode of action.

My results also show that, compared with unstimulated or basal levels of TGF-α secretion, the presence of this growth factor in secreted form is augmented by exogenous PMA, FBS and EGF to an extent that is statistically significant at a 5% level of confidence or better. The consistency between these results and those reported in the literature is described in section 2.3.2 and need not be repeated here. Regarding PMA, however, it is of interest to note that the rapid nature of the cleavage response suggests that thi PKC-dependent mechanism does not rely upon the relatively time-consuming synthesis of pro-TGF-α but rather upon activation of some essential cleavage enzyme, increased exposure of pro-TGF-α to this enzyme or its conversion to a better substrate for the enzyme. It is also noteworthy that exogenous EGF appreciably and autoinductively increases the accumulation of TGF-α in the media conditioned by the A431 and WHCO cell lines, particularly WHCO-5. These results necessarily involve the relatively lengthy process of TGF-α gene expression but also imply an enhanced cleavage efficiency, which may well be linked to PKC activation.

It is surprising to observe from the immunoprobe analysis of unstimulated and stimulated cells that the intensities of the bands appear to be independent of stimulation. Unlike FBS, PMA and EGF apparently inhibit differential glycosylation. In the case of meso-TGF-α

species, they may favour cleavage at the lys^{96}-lys^{97} cleavage site.

5.2.2 Cell proliferation and colony formation assays

The A431 and WHCO cell lines secrete TGF-α constitutively and to overexpress EGF-Rs. Cell proliferation and colony formation assays were carried out to determine the effects of exogenous TGF-α on the proliferative and migratory behaviour of the cell lines *in vitro*.

The proliferation profiles of the subject cell lines in response to exogenous TGF-α vary appreciably (Figure 2.9). As noted in section 2.3.3, the profiles for A431, WHCO-1 and WHCO-5 are consistent with results reported in the literature. In the latter case, it is suggested that the decline in proliferation from 0.1ng/ml onwards may be attributable to increasing down-regulation and desensitisation of the EGF-R.

From the colony formation assays (Figure 2.10 and Table V) it would appear that exogenous TGF-α inhibits the formation of colonies in the A431 cell line (as reported elsewhere) and WHCO-5 cell line but promotes it in the WHCO-1 cell line. Regarding WHCO-3, the addition of TGF-α yields fewer but larger and more diffuse colonies. This result, which is suggestive of cell migration, is in accordance with some of my earlier research (Jones 1989).

5.3 The epidermal growth factor receptor (Chapter 3)

In Thornley and Jones (1992), it was proposed that the unusually short doubling time of the WHCO-5 cell line may be partly the result of a

signalling defect involving overexpression of TGF-α and low-affinity EGF-Rs. The principal aim of the experiments described in Chapter 3 was to determine the hitherto unknown number and affinity of EGF-Rs in this cell line.

Table VI shows the number of EGF-Rs per cell and dissociation constants for the A431 and WHCO cell lines. The values obtained for the A431 and WHCO-3 cell lines are in good agreement with those reported in the literature; those for the WHCO-5 cell line lend support to the abovementioned proposal in Thornley and Jones (1992).

Hyperproduction of EGF-Rs has been described as the hallmark of SCCs. Overexpression of low-affinity EGF-Rs has recently been noted in a number of oesophageal SCC cell lines. The results of the present study are congruent with this literature and serve to confirm and extend those reported in Veale and Thornley (1989).

5.4 Gene amplification (Chapter 4)

The WHCO cell lines, especially WHCO-5, constitutively synthesise and secrete relatively high levels of TGF-α; they also prod[illegible]ernumary low-affinity EGF-Rs. The aim of Chapter 4 was to determine whether these phenomena are associated with amplification of the TGF-α and EGF-R genes.

Estimates of gene amplification in the subject cell lines are given in Table VIII. As may be seen, there is no evidence of TGF-α gene amplification, a result that is in accordance with the literature. On the other hand, amplification of the EGF-R gene, which is known to occur in A431 cells, is also found to be a pronounced characteristic of the

WHCO cells. This result is not surprising in the light of other research into primary oesophageal tumours and derived cell lines. Moreover, there is in the WHCO cell lines a strong positive correlation between EGF-R gene amplification and protein overexpression (Table IX).

5.5 Conclusions

There is extensive evidence in the literature that TGF-α interacts with the EGF-R by means of an autocrine pathway that is regulated in normal but deregulated in neoplastic cells, and in the latter confers a growth advantage that is important to tumour progression. In general, overproduction of an autocrine growth factor is not sufficient for malignant transformation. Thus, although Fischer rat fibroblasts are transformed *in vitro* following transfection with a human TGF-α cDNA expression vector (Rosenthal *et al.*, 1986), overexpression of TGF-α in skin keratinocytes results only in the formation of hyperplastic epidermis but not of tumours (Finzi *et al.*, 1988). Complete transformation appears to require at least a second defect in the autocrine signalling pathway. Therefore one would expect neoplastic cells to display overproduction of an autocrine mitogen coupled with a defect in one or more of the mechanisms which regulate the activity of its receptor.

The summary of findings set out in sections 5.2 to 5.4, which accords with the observations in the preceding paragraph, would appear to indicate that TGF-α and its receptor interact via an amplified autocrine pathway in certain oesophageal SCCs of the WHCO series. First, it has been demonstrated that the subject WHCO cell lines not only constitutively produce and secrete significant quantities of TGF-α but also concomitantly overexpress low-affinity EGF-Rs. These results are

in accordance with the suggestion in Cutry *et al.* (1988) that TGF-α may be primarily dependent on this class of EGF-R for eliciting its mitogenic effect. Secondly, the seemingly novel identification of cell-associated meso-TGF-α is indicative of an autocrine (or intracrine) pathway. Finally, the ability of exogenous EGF to significantly enhance the accumulation of secreted TGF-α points to the existence of a functional autoinductive pathway. These findings support the conclusions concerning an autocrine role for TGF-α reached in various other studies of oesophageal SCCs, such as Yoshida *et al.* (1990).

In general, TGF-α acts to promote cellular proliferation and migration and, *in vivo*, tumour expansion, metastasis and neovascularisation. The aforementioned proliferation and colony formation assays were designed to examine the effects of exogenous TGF-α on proliferation and migration in the subject cell lines. The resulting proliferation profiles were varied, as is often found in the literature, but in all cases exogenous TGF-α stimulated proliferation above basal levels. Exogenous TGF-α had no uniform effect on colony formation in the subject cell lines; the fewer but larger and more diffuse colonies observed in the WHCO-3 cell line are suggestive of cell migration. These results are consistent with TGF-α's well-known role as a mitogenic factor.

It should be noted that most oesophageal carcinoma tissues obtained from surgical specimens express higher levels of TGF-α and EGF-R than does normal oesophageal tissue (Yoshida *et al.*, 1990). This encourages one to believe that the results and conclusions reported in this study are not mere *in vitro* artifacts but are likely to have relevance in the progression of primary tumours such as those from which the WHCO cell lines were derived.

5.6 Thoughts on future research

It is desirable that the experiments that form the basis of this study be repeated in the context of an improved experimental design. It is clearly important to establish the reasons for the constitutive secretion of TGF-α by the subject cell lines. *Inter alia*, this may be attributable to rearrangement of the TGF-α gene sequence, which Southern blot analysis would help to clarify, or to the action of some oncoprotein (such as p21), the presence of which could be detected by Western blot analysis. Northern blot analysis could be used to test the validity of my speculation that the unusually high constitutive secretion of TGF-α by the WHCO-5 cell line may be ascribable to overexpression and enhanced stability of messenger transcript. As regards the EGF-R, it would be of interest to refine the analysis of receptor affinity by the use of antibodies directed specifically at the supernumary low-affinity class known to be present: only thus would it be possible to determine whether or not a relatively small number of high-affinity receptors is also present. As regards EGF-R gene amplification, the use of Southern blot analysis would shed light on whether the receptor gene sequence is rearranged, which would be indicative of the presence of aberrant mRNA transcripts and protein products. In particular, my conclusion that there is an autocrine relationship between TGF-α and the EGF-R in the subject cell lines needs to be exposed to the rigorous proof that would be afforded by the use of appropriate antisense constructs or specific antibodies in blocking experiments such as those described in Stromberg *et al.* (1992).

Like the present study, the above outline of directions for further research focuses narrowly on aspects of the TGF-α/EGF-R autocrine pathway. But tumourigenesis is a complex, multi-step process, and therefore one must keep in mind that other mechanisms that impinge

on this pathway may reinforce it when deregulated. Work is being undertaken on the secretion and action of TGF-β in the WHCO cell lines, and this is of importance in the present context because, as mentioned in section 1.3.1 of Chapter 1, TGF-β modulates the effects of TGF-α. Also relevant is the overexpression of c-Myc by the WHCO-3 cell line (Heiss 1992) in view of the fact, discussed in section 1.3.3 of Chapter 1, that transcription factors have an important role in controlling the functional activity of growth factors in mutually interactive autocrine loops. In evaluating the results of the proposed research programme, it would be necessary to take account of the findings that may emerge from such parallel lines of enquiry.

Appendix A

A1 Phosphate buffered saline (pH 7.6)

137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM $Na_2HPO_4.12H_2O$. Make up with Milli-Q water and filter sterilise. For routine laboratory use make up with distilled water.

A2 Radioimmunoassays

A2.1 Radioimmunoassay methodology

Reconstituted conditioned medium samples (100µl) were placed in 12x75-mm plastic tubes and incubated at 4°C with 50µl per tube of sheep anti-human TGF-α antiserum for 24 hours. One hundred microlitres of ^{125}I-TGF-α were added to each tube and the mixtures left for a further 24 hours at 4°C. On day three, 100µl of precipitating donkey anti-sheep antiserum were added to each tube and the mixtures incubated for 15 minutes at room temperature. After the addition of 1.5ml of chilled TBS (pH 8.5), the mixtures were vortexed, centrifuged at 2500rpm for 15 minutes in an MSE bench-top centrifuge and their supernatants decanted. The radioactivity present in the immunoglobulin pellets was determined by counting for 2 minutes in a Packard Cobra auto-gamma counter. The assay's sensitivity and half-maximal displacement were quoted as 0.02ng TGF-α/tube and 1.7ng/ml TGF-α respectively.

A2.2

Table A1 Radioimunoassay 1 - data and statistical analysis

Point	dpm	sdpm	α (%)	β	N_e	C	Test	
							T	V
NSB	270.47	0.00						
B_o	9336.22	9065.75	100.00					
S1	8914.97	8644.50	95.35					
S2	7390.10	7119.63	78.53					
S3	6049.72	5779.25	63.75					
S4	2062.47	1792.00	19.77					
S5	1699.72	1429.25	15.77					
SFM	9251.60	8981.13	99.07	*				
SFCM(A)	8750.60	8480.13	93.54	0.037	$2.05x10^7$	3.6	t	4.01
SFCM(3)	8604.47	8334.00	91.93	0.048	$9.86x10^6$	9.7	t	4.42
SM	9396.97	9126.50	100.67	*				
SFCM(A)	8750.60	8480.13	93.54	0.037	$2.05x10^7$	3.6		
SsCM(A)	8024.97	7754.50	85.54	0.088	$2.71x10^7$	6.5	U	1.4%
SFCM(3)	8604.47	8334.00	91.93	0.048	$9.86x10^6$	9.7		
SsCM(3)	8301.10	8030.63	88.58	0.068	$1.02x10^7$	13.4	t	2.35

NSB = non-specific ^{125}I-TGF-α binding; B_o = maximum ^{125}I-TGF-α binding; **S(1-5)** = standard TGF-α concentrations, where **S1** = 0.025ng/ml, **S2** = 0.140ng/ml, **S3** = 0.250ng/ml, **S4** = 1.380ng/ml, **S5** = 2.500ng/ml; **SFM** = serum-free medium (DMEM/F12, 3:1); **SFCM(A)** = unstimulated serum-free conditioned medium, A431; **SFCM(3)** = unstimulated serum-free conditioned medium, WHCO-3; **SM** = unconditioned serum-containing medium (DMEM/F12 + 10%FBS); **SsCM(A)** = serum-stimulated conditioned medium, A431; **SsCM(3)** = serum-stimulated conditioned medium, WHCO-3; **dpm** = average number of disintegrations per minute; **sdpm** = average number of specific disintegrations per minute (i.e. dpm - 270.47); α (%) = [sdpm/9065.75] x 100; β = concentration (ng/ml) of TGF-α in 10x concentrated medium samples [= (2-logα)/0.777]; * = concentration below assay sensitivity; N_e = number of cells conditioning 20ml of unconcentrated medium; **C** = quantity of TGF-α secreted in 20 hours in units of pg/10^6 cells; **T** = type of statistical test, t representing Student's t test and U the Mann-Whitney test; **V** = value of test. The U values are a precise measure of the level of confidence on a one-tailed basis. At a 5% level of confidence, the critical value of Student's t for a one-tailed test with six degrees of freedom is ± 1.94. **Note:** The correlation coefficient and standard error of the above linear regression equation [β=(2-logα)/0.777] are -0.999 and $2.566x10^{-3}$ respectively.

Table A2 Radioimmunoassay 2 - data and statistical analysis

Point	dpm	sdpm	α (%)	β	N_o	C	Test	
							T	V
NSB	233.60	0.00						
B_o	5963.22	5729.62	100.00					
S1	5533.72	5300.12	92.50					
S2	4600.72	4367.12	76.22					
S3	3813.97	3580.37	62.49					
S4	999.35	765.75	13.36					
S5	762.22	528.62	9.23					
SFM	5775.47	5541.87	96.72	*				
SFCM(A)	5486.47	5252.87	91.68	0.032	$1.75x10^7$	3.6	t	3.46
SFCM(1)	5289.72	5056.12	88.25	0.053	$1.96x10^7$	5.4	U	1.4%
SFCM(3)	5558.72	5325.12	92.94	0.024	$5.53x10^6$	8.6	U	1.4%
SFCM(3)	5075.72	4842.12	84.51	0.078	$1.86x10^7$	8.4	t	7.41
SFCM(5)	5129.10	4895.50	85.44	0.072	$9.80x10^5$	146	t	8.15
SFCM(A)	5486.47	5252.87	91.68	0.032	$1.75x10^7$	3.6		
PsCM(A)	5366.85	5133.25	89.59	0.045	$1.69x10^7$	5.3	t	2.26
SFCM(3)	5558.72	5325.12	92.94	0.024	$5.53x10^6$	8.6		
PsCM(3)	5324.60	5091.00	88.85	0.049	$5.53x10^6$	17.7	t	6.50
SFCM(A)	5486.47	5252.87	91.68	0.032	$1.75x10^7$	3.6		
EsCM(A)	4693.60	4460.00	77.84	0.126	$2.02x10^7$	12.5	U	1.4%
SFCM(1)	5289.72	5056.12	88.25	0.053	$1.96x10^7$	5.4		
EsCM(1)	5165.72	4932.12	86.08	0.068	$2.11x10^7$	6.4	t	3.52
SFCM(3)	5075.72	4842.12	84.51	0.078	$1.86x10^7$	8.4		
EsCM(3)	4792.22	4558.62	79.56	0.113	$1.91x10^7$	11.8	t	4.06
SFCM(5)	5129.10	4895.50	85.44	0.072	$9.8x10^5$	146.0		
EsCM(5)	1591.22	1357.62	23.69	1.014	$1.4x10^6$	1449	t	67.3

NSB = non-specific ^{125}I-TGF-α binding; B_o = maximum ^{125}I-TGF-α binding; **S(1-5)** = standard TGF-α concentrations, where **S1** = 0.025ng/ml, **S2** = 0.140ng/ml, **S3** = 0.250ng/ml, **S4** = 1.380ng/ml, **S5** = 2.500ng/ml; **SFM** = serum-free medium (DMEM/F12, 3:1); **SFCM(A)** = unstimulated serum-free conditioned medium, A431; **SFCM(1)** = unstimulated serum-free conditioned medium, WHCO-1; **SFCM(3)** = unstimulated serum-

free conditioned medium, WHCO-3; **SFCM(5)** = unstimulated serum-free conditioned medium, WHCO-5; **PsCM(A)** = PMA-stimulated conditioned medium, A431; **PsCM(3)** = PMA-stimulated conditioned medium, WHCO-3; **EsCM(A)**= EGF-stimulated serum-free conditioned medium, A431; **EsCM(1)** = EGF-stimulated serum-free conditioned medium, WHCO-1; **EsCM(3)** = EGF-stimulated serum-free conditioned medium, WHCO-3; **EsCM(5)** = EGF-stimulated serum-free conditioned medium, WHCO-5; **dpm** = average number of disintegrations per minute; **sdpm** = average number of specific disintegrations per minute (i.e. dpm - 233.60); α (%) = [sdpm/5729.62] x 100; β = concentration (ng/ml) of TGF-α in 10x concentrated medium samples [= (1.986-logα)/0.755]; * = concentration below assay sensitivity; N_c = number of cells conditioning 20ml of unconcentrated medium; **C** = quantity of TGF-α secreted in 20 hours in units of pg/10^5 cells; **T** = Statistical test, t representing Student's t test and U the Mann-Whitney test; **V** = value of test. The U values are a precise measure of the level of confidence on a one-tailed basis. At a 5% level of confidence, the critical value of Student's t for a one-tailed test with six degrees of freedom is ± 1.94. **Note:** The correlation coefficient and standard error of the above linear regression equation [β=(1.986-logα)/0.755] are -1.00 and 1.362×10^{-3} respectively.

A3 Detachment solution

1.25ml PMSF/methanol stock, 2.50ml trasylol stock. Make up to 250ml with PBS.

(PMSF/methanol stock: 34.8mg PMSF in 10ml methanol; trasylol stock: 14mg trasylol in 10ml PBS).

A4 Electrophoresis sample buffer (pH 6.8)

1ml glycerol, 0.5ml β-mercaptoethanol, 0.3g SDS, 0.15g Tris. Make up to 10ml with distilled water.

A5 SDS polyacrylamide gel electrophoresis

A5.1 10-20% gradient gels

Stacking gel (5% acrylamide): 0.48g acrylamide, 2.5ml stacking buffer solution (0.5M Tris, pH 6.8), 0.4ml SDS solution (50mg/ml), 0.55ml bis-acrylamide solution (25mg/ml). Make up to 10ml with distilled water. Add 32µl TEMED and 130µl 1.25% ammonium persulphate solution (APS).

Separating gel (10% acrylamide): 0.6g acrylamide, 1.5ml separating buffer solution (1.5M Tris, pH 8.8), 0.24ml SDS solution, 0.24ml bis-

acrylamide solution. Make up to 6ml with distilled water. Add 18µl TEMED and 80µl APS.

Separating gel (20% acrylamide): 1.2g acrylamide, 1.5ml separating buffer solution, 0.24ml SDS solution, 0.24ml bis-acrylamide solution, 1.5ml 20% glycerol. Make up to 6ml with distilled water. Add 18µl TEMED and 80µl APS.

A5.2 Running buffer (pH 8.3-8.4)

2.42g Tris, 0.80g SDS, 11.56g glycine. Make up to 800ml with distilled water.

A5.3 Transfer buffer (pH 8.3)

42.3g glycine, 9.0g Tris, 600ml methanol. Make up to 3 litres with distilled water.

A6 Blocking buffer (pH 8.0)

0.606g Tris, 4.383g NaCl, 0.25ml Tween-20, 2.5g non-fat milk powder. Make up to 500ml with distilled water.

A7 TBS Tween-20 (pH 8.0)

0.606g Tris, 4.383g NaCl, 0.25ml Tween-20. Make up to 500ml with distilled water.

A8 Antibodies

A8.1 Primary

A8.1.1 Polyclonal anti-human TGF-α (Biomedical Technologies Inc.)

This highly specific sheep polyclonal antiserum was raised against human TGF-α and does not cross-react under radioimmunoassay conditions with either recombinant rat TGF-α or human EGF. The antibody was diluted 1:2.5 in TBST for use as a primary immunoprobe.

A8.1.2 M[illegible]onal anti-human TGF-α (Oncogene Science)

This mouse monoclonal antibogy (IgG_1) was produced by immunising mice with recombinant human TGF-α. The antibody reacts with native and denatured forms of rat and human mature TGF-α but shows no cross-reactivity with human or mouse EGF. The antibody was diluted in TBST and used as a primary immunoprobe at a concentration of 10μg/ml.

A8.2 Secondary

A8.2.1 Polyclonal donkey anti-sheep (The Binding Site)

The donkey anti-sheep peroxidase-conjugated (heavy and light chain specific) IgG stock solution was diluted in TBST and used at a concentration of 1:200.

A8.2.2 Polyclonal goat anti-mouse (Sigma)

The goat anti-mouse peroxidase-conjugated (Fab specific) IgG stock solution was diluted in TBST and used at a concentration of 1:100.

A9 4-chloro-1-napthol substrate solution

2ml 4-chloro-1-napthol stock, 60μl hydrogen peroxide, 48 ml PBS.

(4-chloro-1-napthol stock: 0.15g 4-chloro-1-napthol. Make up to 50 ml with methanol.)

Appendix B

Table B1 A431 ^{125}I-EGF competitive binding data

P_n	α	β	V_t	dpm	sdpm
1	.77	0	914.5	22665	16827
2	.77	90	914.5	5838	0
3	.77	0.01	914.5	25845	20007
4	.77	0.1	914.5	24423	18585
5	.77	0.5	914.5	22524	16686
6	.77	1.0	914.5	22670	16832
7	.77	6.0	914.5	18192	12354
8	.77	10.0	914.5	16539	10701
9	.77	15.0	914.5	13645	7807

P_n = point number; α = total amount (ng) of ^{125}I-EGF per point; β = total amount (ng) of receptor grade EGF per point; V_t = total volume of competitive binding solution per point; **dpm** = average number of disintegrations per minute; **sdpm** = average number of specific disintegrations per minute (i.e. **dpm** - 5838); volume of competitive binding solution added per well = 290 µl; number of cells per well= 1.31×10^5; % non-specific binding (i.e. [**dpm** for point number 2/**dpm** for point number 1] x 100) = 25.76%.

Table B2 WHCO-3 ^{125}I-EGF competitive binding data

P_n	α	β	V_t	dpm	sdpm
1	.81	0	915	24698	21888
2	.81	90	915	2810	0
3	.81	0.01	915	25098	22288
4	.81	0.10	915	25228	22418
5	.81	0.5	915	23880	21070
6	.81	1.0	915	23271	20461
7	.81	2.0	915	21368	18558
8	.81	6.0	915	13872	11062
9	.81	10.0	915	11738	8928
10	.81	15.0	915	10490	7680

P_n = point number; α = total amount (ng) of ^{125}I-EGF per point; β = total amount (ng) of receptor grade EGF per point; V_t = total volume of competitive binding solution per point; **dpm** = average number of disintegrations per minute; **sdpm** = average number of specific disintegrations per minute (i.e. **dpm** - 2810); volume of competitive binding solution added per well = 296 µl; number of cells per well= 1.35×10^5; % non-specific binding (i.e. [**dpm** for point number 2/**dpm** for point number 1] x 100) = 11.38%.

Table B3 WHCO-5 ^{125}I-EGF competitive binding data

P_n	α	β	V_t	dpm	sdpm
1	.81	0	915	13286	11787
2	.81	90	915	1499	0
3	.81	.1	915	13227	11728
4	.81	0.5	915	12067	10568
5	.81	1.0	915	7041	5542
6	.81	6.0	915	6758	5259
7	.81	10.0	915	5350	3851
8	.81	15.0	915	4428	2929
9	.81	20.0	915	3704	2205

P_n = point number; α = total amount (ng) of ^{125}I-EGF per point; β = total amount (ng) of receptor grade EGF per point; V_t = total volume of competitive binding solution per point; dpm = average number of disintegrations per minute; sdpm = average number of specific disintegrations per minute (i.e. dpm - 1499; volume of competitive binding solution added per well = 300 µl; number of cells per well= 1.13×10^5; % non-specific binding (i.e. [dpm for point number 2/dpm for point number 1] x 100) = 11.28%.

Appendix C

C1 Luria-Bertani medium (pH 7.6)

1% tryptone; 0.5% yeast extract; 1% NaCl. Make up in autoclaved distilled water.

C2 Large scale preparation of plasmid DNA

Bacterial transformants were harvested by centrifugation at 6000rpm (DuPont RC-5 Sorvall centrifuge) for 10 minutes. The bacterial cell pellets were resuspended in 5ml of Solution I (see section C2.1) and incubated at room temperature for 10 minutes. The cells were lysed by the addition of 10ml of Solution II (see section C2.2), and their linear DNAs digested at room temperature for 15 minutes. Next, 7.5ml of Solution III (see section C2.3) were added and the bacterial proteins precipitated on ice for a period of 10 minutes. The cellular debris was pelleted by centrifugation at 16000 rpm and 4°C for 15 minutes, and the undigested DNAs precipitated for 15 minutes following the addition of 0.6 volumes of isopropanol. The precipitated DNAs were isolated by centrifuging for 10 minutes at 15000rpm and 25°C, rinsed with 5ml of ethanol, vacuum-dried (Sarvant Speedvac concentrator) for 15 minutes, and resuspended at 45°C over a period of 2 hours in 4ml of Tris-EDTA (TE; see section C2.4). Following the addition of 4g of caesium chloride (CsCl) and 0.4ml of a 10mg/ml ethidium bromide (EtBr) solution, the refractive index of the mixture was adjusted to 1.389 using an Atago refractometer. The mixture was aliquoted into Beckmann Quickseal tubes and ultracentrifuged (Beckmann L7-65 ultracentrifuge) at 45000rpm and 10°C for 16 hours using a VTi65.2 rotor. The supercoiled plasmid DNA was carefully aspirated through an 18-gauge needle and aliquoted into Eppendorf tubes. Finally, the intercalated EtBr was phase-extracted 3 to 4 times with an equal volume of Tris-saturated

butanol (0.1M Tris/butanol, 1:1), and the CsCl removed from solution by dialysing at room temperature for 4 hours in 2 changes of autoclaved distilled water.

C2.1 Solution I (pH 8.0)

50mM glucose; 25mM Tris; 10mM EDTA. Make up in autoclaved distilled water. Add 5mg of lysozyme (Sigma) per ml of solution.

C2.2 Solution II

0.2M NaOH; 10% SDS. Make up in autoclaved distilled water.

C2.3 Solution III (pH 5.5)

5M potassium acetate; 11ml glacial acetic acid. Make up to 100 ml in autoclaved distilled water.

C2.4 TE (pH 8.0)

10mM Tris; 10mM EDTA-$Na_2.2H_2O$. Make up in autoclaved distilled water.

C3 Salt extraction of genomic DNA

The cell monolayers were briefly rinsed in PBS and harvested by scraping in 1ml of STE (see section C3.1) per tissue culture dish. The cells were subsequently lysed in 1% SDS. Proteinase K (Boehringer Mannheim) was added to a final concentration of 2mg/ml and the mixture incubated at 55°C for 16 hours. The cellular proteins were precipitated by the addition of 500µl (per ml of STE) of a 6M NaCl solution, and pelleted at 3000rpm (DuPont RC-5 Sorvall centrifuge) for 20 minutes. The genomic DNA was precipitated with 2.5 volumes of absolute ethanol, spooled out of solution and rinsed with 70% ethanol. The DNA was vacuum-dried (Savant Speedvac concentrator) for 20 minutes and finally resuspended overnight at 45°C in 0.5ml of

autoclaved distilled water.

C3.1 STE (pH 8.0)

0.1M NaCl; 0.05M Tris; 1mM EDTA-$Na_2.2H_2O$. Make up in autoclaved distilled water.

C4

Table C1 Spectrophotometric determination of genomic DNA concentration.

Cell line	OD_{260nm}	µg/ml[1]	µg/µl[2]
A431	0.202	10.1	1.01
WHCO-1	0.132	6.6	0.66
WHCO-3	0.346	17.3	1.73
WHCO-5	0.174	8.7	0.87

1 One OD_{260nm} unit = 50µg/ml double stranded DNA.
2 Concentration of neat genomic DNA (sample analysed was diluted 100 times).

C5 0.8% TAE agarose gel

1.25ml of 40x TAE running buffer; 2.5µl of a 10mg/ml EtBr solution; 0.4g agarose. Make up to 50ml with autoclaved distilled water.

C5.1 40x TAE running buffer (pH 7.2)

1.6M Tris; 0.8M Na acetate.$3H_2O$; 40mM EDTA-$Na_2.2H_2O$. Make up in autoclaved distilled water.

C6 Purification of gene inserts

The TGF-α and β-globin inserts were excised from the TAE agarose gels, weighed and dissolved in approximately 3 volumes of NaI for 10 minutes at 55°C. The inserts were allowed to bind to a glassmilk suspension (1µl suspension/µg DNA) for 15 minutes on ice. The glassmilk/DNA complexes were pelleted by microfuging (Eppendorf 5413 microfuge) for

5 minutes and washed 3 times with 50 volumes of ice-cold New Wash solution. The washed pellets were resuspended in an equal volume of autoclaved distilled water and the DNAs solubilised at 55°C for 2 to 5 minutes. The glassmilk suspensions were pelleted by microfuging for 30 seconds and the supernatants containing the eluted DNAs carefully removed and stored at -20°C.

C7 0.8% TBE agarose gel

2.5ml of 20x TBE running buffer; 5µl of a 10mg/ml EtBr solution; 0.4g agarose. Make up to 50ml with autoclaved distilled water.

C7.1 20x TBE running buffer (pH 8.2)

1M Tris; 1M boric acid; 20mM EDTA-$Na_2.2H_2O$. Make up in autoclaved distilled water.

C7.2 DNA loading buffer (5x)

50% glycerol; 20% 0.5M EDTA-$Na_2.2H_2O$; 5ml TBE running buffer; 10% bromophenol blue. Make up to 100ml with autoclaved distilled water. Add 2.0µl to DNA samples prior to loading.

C8

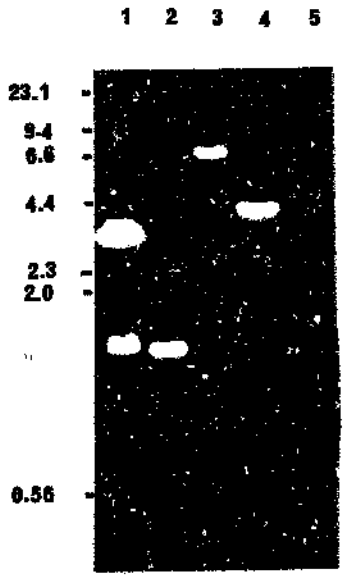


Figure C1 0.8% TBE agarose gel showing restricted plasmids and purified inserts. Lane 1, 1.3kb full-length TGF-α cDNA insert and the 3.5kb plasmid vector; lane 2, purified TGF-α insert; lane 3, linearised 7.0kb EGF-R plasmid; lane 4, 0.9kb β-globin intervening sequence II and the 4kb pBR322 plasmid vector; lane 5, purified β-globin insert. The molecular sizes (kb) of the λ II DNA markers are indicated on the left.

C9

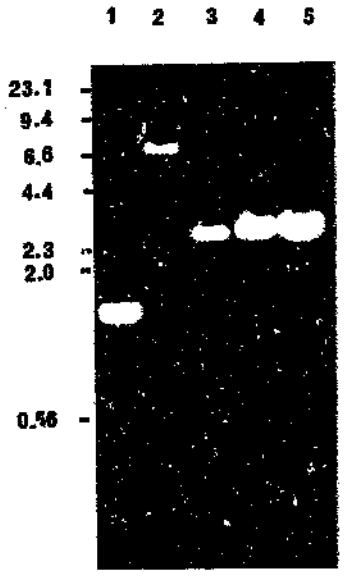


Figure C2 Probe concentration assessement by dilution analysis. 0.6% TBE agarose gel showing: lane 1, unknown quantity of the purified 1.3kb TGF-α cDNA insert; lane 2, unknown quantity of the 7.0kb linearised EGF-R plasmid; lanes 3 to 5 - 22.5ng, 45ng and 90ng of a linearised 2.7kb pUC18 plasmid vector. The molecular sizes of the λ II DNA markers are indicated on the left.

C10 Multiprime labelling of DNA probes

25 to 100ng of DNA probe was denatured at 100°C for 5 minutes. The denatured probe was added to a reaction mixture comprising: 4µl each of dCTP, dTTP and dGTP; 5µl each of labelling buffer, primer and dATP 5'-[α^{32}P] triphosphate (3000 Ci/mmol, Amersham); and 2µl of Klenow enzyme. Autoclaved distilled water was added to give a final volume of 50µl. The labelling reaction was carried out at 37°C for a period of 4 hours. The percentage of dATP 5'-[α^{32}P] triphosphate incorporated into the probe was determined at the end of this period by spotting 1µl of reaction mixture onto each of two glass fibre discs. The discs were placed

in scintillation vials and the β emission counted by Cerenkov analysis in a 1900CA Packard liquid scintillation analyser. The glass fibre discs were washed under vacuum filtration with ice cold 10% trichloroacetic acid and their β emission recounted.

C10.1 Specific activity of labelled probe

Specific activity of labelled probe = $[\kappa/\tau]$ x 10^3 dpm/μg; where κ represents the amount of radioactive incorporation (dpm), and τ represents the total amount of probe DNA (ng).

κ = total μCi x 2.2×10^4 x percentage incorporation.

τ = [Total μCi x 13.2 x percentage incorporation]/[number of dNTPs x their average specific activity] + original amount of probe DNA (ng).

C11 Prehybridisation solution

10ml distilled water; 10ml of 50x Denhardt's solution (see section C11.1); 25ml of 20x SSC (see section C11.2); 2ml of PB (see section C11.3); 3ml of a 10 mg/ml herring sperm DNA solution; 50ml of deionised formamide.

C11.1 50x Denhardt's solution

1% ficoll; 1% polyvinylpyrrolidone; 0.5% bovine serum albumin. Make up in autoclaved distilled water.

C11.2 20x Standard saline citrate

3.0M NaCl; 0.3M tri-sodium citrate. Make up in autoclaved distilled water.

C11.3 Phosphate buffer (pH 6.56)

1M $Na_2HPO_4.12H_2O$; 1M $NaH_2PO_4.2H_2O$. Make up in autoclaved distilled water.

References

Aaronson, S.A. (1991). Growth factors and cancer. *Science* 254, 1146-1153.

Alitalo, K. and Schwab, M. (1986). Oncogene amplification in tumour cells. *Advances in Cancer Research* 47, 235-282.

Anklesaria, P., Teixidó, J., Laiho, M., Pierce, J.H., Greenberger, J.S. and Massagué, J. (1990). Cell-cell adhesion mediated by binding of membrane anchored transforming growth factor α to epidermal growth factor receptors promotes cell proliferation. *Proceedings of the National Academy of Sciences of the U.S.A.* 87, 3289-3293.

Anzano, M., Roberts, A.B., Smith, J.M., Sporn, M.B. and De Larco, J.E. (1983). Sarcoma growth factor from conditioned medium of virally transformed cells is composed of both type α and type β transforming growth factors. *Proceedings of the National Academy of Sciences of the U.S.A.* 80, 6264-6268.

Avivi. A., Lax, I., Ullrich, A., Schlessinger, J., Givol, D. and Morse, B. (1991). Comparison of EGF receptor sequences as a guide to study the ligand binding site. *Oncogene* 6, 673-676.

Baccarini, M., Gill, G.N. and Stanley, E.R. (1991). Epidermal growth factor stimulates phosphorylation of RAF-1 independently of receptor autophosphorylation and internalisation. *The Journal of Biological Chemistry* 266, 10941-10945.

Bade, E.G. and Fiendler, S. (1988). Liver epithelial cell migration

induced by epidermal growth factor or transforming growth factor alpha is associated with changes in the gene expression of secreted proteins. *In Vitro Cellular and Developmental Biology* 24, 149-154.

Banks-Schlegel, S.P. and Quintero, J. (1986). Human oesophageal carcinoma cells have fewer, but higher affinity epidermal growth factor receptors. *The Journal of Biological Chemistry* 261, 4359-4362.

Barrandon, Y. and Green, H. (1985). Cell migration is essential for sustained growth of keratinocyte colonies: The roles of transforming growth factor-α and epidermal growth factor. *Cell* 50, 1131-1137.

Barrett-Lee, P., Travers, M., Luqmani, Y. and Coombes, R.C. (1990). Transcripts for transforming growth factors in human breast cancer: clinical correlates. *British Journal of Cancer* 61, 612-617.

Barton, C.M., Hall, P.A., Hughes, C.M., Gullick, W.J. and Lemoine, N.R. (1991). Transforming growth factor alpha and epidermal growth factor in human pancreatic cancer. *The Journal of Pathology* 163, 111-116.

Bellot, F., Moolenaar, W., Kris, R., Mirakhur, B., Verlaan, I., Ullrich, A., Schlessinger, J. and Felder, S. (1990). High-affinity epidermal growth factor binding is specifically reduced by a monoclonal antibody, and appears necessary for early responses. *The Journal of Cell Biology* 110, 491-502.

Berkers, J.A.M., van Bergen en Henegouwen, P.M.P. and Boonstra, J.

(1992). The effects of receptor density and cell shape on epidermal growth factor binding. *Journal of Receptor Research* 12, 71-100.

Bishop, J.M. (1991). Molecular themes in oncogenesis. *Cell* 64, 235-248.

Bissonnette, F., Cook, C., Geoghegan, T., Steffen, M., Henry, J., Yussman, M.A. and Schultz, G. (1992). Transforming growth factor-α and epidermal growth factor messenger ribonucleic acid and protein levels in human placentas from early, mid, and late gestation. *American Journal of Obstetrics and Gynaecology* 166, 192-199.

Bjorge, J.D., Paterson, A.J. and Kudlow, J.E. (1989). Phorbol ester or epidermal growth factor (EGF) stimulates the concurrent accumulation of mRNA for the EGF receptor and its ligand transforming growth factor-α in a breast cancer cell line. *The Journal of Biological Chemistry* 264, 4021-4027.

Brachmann, R., Lindquist, P.B., Nagishima, M., Kohr, W., Lipari, T., Napier, M. and Derynck, R. (1989). Transmembrane TGF-α precursors activate EGF/TGF-α receptors. *Cell* 56, 691-700.

Bradshaw, E. and Harington, J.S. (1987). The problem of oesophageal cancer relative to other cancers in South Africa 1949-1979. *South African Medical Journal* (supplement), 1-2.

Bringman, T.S., Lindquist, P.B. and Derynck, R. (1987). Different transforming growth factor-α species are derived from a glycosylated and palmitoylated transmembrane precursor. *Cell* 48, 429-440.

Cadena, D.J. and Gill, G.N. (1992). Receptor tyrosine kinases. *Journal of the Federation of American Societies for Experimental Biology* 6, 2332-2337.

Canals, F. (1992). Signal transmission by epidermal growth factor receptor: Coincidence of activation and dimerisation. *Biochemistry* 31, 4493-4501.

Cardinali, M., Sartor, O. and Robbins, K.C. (1992). Suramin, an experimental chemotherapeutic drug, activates the receptor for epidermal growth factor and promotes growth of certain malignant cells. *The Journal of Clinical Investigation* 89, 1242-1247.

Carpenter, G. and Cohen, S. (1990). Epidermal growth factor. *The Journal of Biological Chemistry* 265, 7709-7712.

Carraway, K.L. and Cerione, R.A. (1991). Comparison of epidermal growth factor (EGF) receptor-receptor interactions in intact A431 cells and isolated plasma membranes. *The Journal of Biological Chemistry* 266, 8899-8906.

Chalazonitis, A., Kessler, J.A., Twardzik, D.R. and Morrison, R.S. (1992). Transforming growth factor α, but not epidermal growth factor, promotes the survival of sensory neurons *in vitro*. *The Journal of Neuroscience* 12, 583-594.

Chen, S-C., Chou, C-K., Wong, F-H., Chang, C. and Hu, C-p. (1991). Overexpression of epidermal growth factor and insulin-like growth factor-I receptors and autocrine stimulation in human oesophageal carcinoma cells. *Cancer Research* 51, 1898-1903.

Cheng, K.K., Day, N.E. and Davies, T.W. (1992). Oesophageal cancer mortality in Europe: paradoxical time trend in relation to smoking and drinking. *British Journal of Cancer* 65, 613-617.

Choudry, Y. and Kenny, A.J. (1991). Hydrolysis of transforming growth factor-α by cell-surface peptidases *in vitro*. *Journal of Biochemistry* 280, 57-60.

Coffey, R.J., Derynck, R., Wilcox, J.N., Bringman, T.S., Goustin, A.S., Moses, H.L. and Pittelkow, M.R. (1987). Production and auto-induction of transforming growth factor-α in human keratinocytes. *Nature* 328, 817-820.

Coffey, R.J., Sipes, N.J., Bascom, C.C., Graves-Deal, R., Pennington, C.Y., Weissman, B.E. and Moses, H.L. (1988). Growth modulation of mouse keratinocytes by transforming growth factors. *Cancer Research* 48, 1596-1602.

Coffey, R.J., Graves-Deal, R., Dempsey, P.J., Whitehead, R.H. and Pittelkow, M.R. (1992). Differential regulation of transforming growth factor α autoinduction in a nontransformed and transformed epithelial cell. *Cell Growth and Differentiation* 3, 347-354.

Countaway, J.L., Nairn, A.C. and Davis, R.J. (1992). Mechanism of desensitisation of the epidermal growth factor receptor protein-tyrosine kinase. *The Journal of Biological Chemistry* 267, 1129-1140.

Cowley, G., Smith, J.A., Gusterson, B., Hendler, F. and Ozanne, B. (1984). The amount of EGF receptor is elevated on squamous cell

carcinomas. *Cancer Cells* 1, 5-10.

Cross, M. and Dexter, T.M. (1991). Growth factors in development, transformation and tumourigenesis. *Cell* 64, 271-280.

Cutry, A.F., Kinniburgh, A.J., Twarzdik, D.R. and Wenner, C.E. (1988). Transforming growth factor alpha (TGF-α) induction of c-*fos* and c-*myc* expression in C3H 10T1/2 cells. *Biochemical and Biophysical Research Communications* 152, 216-222.

Decker, S.J. (1990). Epidermal growth factor and transforming growth factor-α induce differential processing of the epidermal growth factor receptor. *Biochemical and Biophysical Research Communications* 166, 615-621.

Decker, S.J., Alexander, C. and Habib, T. (1992). Epidermal growth factor (EGF)-stimulated tyrosine phosphorylation and EGF receptor degradation in cells expressing EGF receptors truncated at residue 973. *The Journal of Biological Chemistry* 267, 1104-1108.

De Fize, L.H.K., Boonstra, J., Meisenhelder, J., Kruijer, W., Tertoolen, L.G.J., Tilly, B.C., Hunter, T., van Bergen en Henegouwen, P.M.P., Moolenaar, W.H. and de Laat, S.W. (1989). Signal transduction by epidermal growth factor occurs through the subclass of high affinity receptors. *The Journal of Cell Biology* 109, 2495-2507.

De Larco, J.E. and Todaro, G.J. (1978). Growth factors from murine sarcoma virus-transformed cells. *Proceedings of the National Academy of Sciences of the U.S.A.* 75, 4001-4005.

Derynck, R., Roberts, A.B., Winkler, M.E., Chen, E.Y. and Goeddel, D.V. (1984). Transforming growth factor-α: precursor structure and expression in *E. coli*. *Cell* 38, 287-297.

Derynck, R. (1987). Structure of transforming growth factors-α and -β and their precursors. In Oncogenes, genes, and growth factors, G. Guroff, ed. (New York: Wiley and Sons Inc.), pp 133-163.

Derynck, R., Goeddel, D.V., Ullrich, A., Gutterman, J.U., Williams, R.D., Bringman, T.S. and Berger, W.H. (1987). Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumours. *Cancer Research* 47, 707-712.

Derynck, R. (1988). Transforming growth factor α. *Cell* 54, 593-595.

Di Fiore, P.P., Pierce, J.H., Fleming, T.P., Hazan, R., Ullrich, A., King, C.R., Schlessinger, J. and Aaronson, S.A. (1987). Overexpression of the human epidermal growth factor receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells. *Cell* 51, 1063-1070.

Ebner, R. and Derynck, R. (1991). Epidermal growth factor and transforming growth factor-α: differential intracellular routing and processing of ligand-receptor complexes. *Cell Regulation* 2, 599-612.

El-Zayat, A.A.E., Pingree, T.F., Mock, P.M., Clark, G.M., Otto, R.A. and Von Hoff, D.D. (1991). Epidermal growth factor receptor amplification in head and neck cancer. *The Cancer Journal* 4, 375-381.

Evarts, R.P., Nakatsukasa, H., Marsden, E.R., Hu, Z. and Thorgeirsson, S.S. (1992). Expression of transforming growth factor-alpha in regenerating liver and during hepatic differentiation. *Molecular Carcinogenesis* 5, 25-31.

Fernandez-Pol, J.A., Klos, D.J. and Hamilton, P.D. (1989). Modulation of transforming growth factor alpha-dependent expression of epidermal growth factor receptor gene by transforming growth factor beta, triiodothyronine, and retinoic acid. *Journal of Cellular Biochemistry* 41, 159-170.

Field, J.A., Reid, R.H., Rieman, D.J., Page Kline, T., Sathe, G., Greig, R.G. and Anzano, M.A. (1992). Structure-function analysis of human transforming growth factor-α by site-directed mutagenesis. *Journal of Biochemistry* 283, 91-98.

Finzi, E., Kilkenny, A., Strickland, J.E., Balashak, M., Bringman, T., Derynck, R., Aaronson, S. and Yuspa, S.H. (1988). TGF-α stimulates growth of skin papillomas by autocrine and paracrine mechanisms but does not cause neoplastic progression. *Molecular Carcinogenesis* 1, 7-12.

Fuller, G.N. and Bigner, S.H. (1992). Amplified cellular oncogenes in neoplasms of the human central nervous system. *Mutation Research* 276, 299-306.

Furuta, Y., Takasu, T., Asai, T., Yoshimura, S., Tokuchi, F., Shinohara, T., Nagashima, K. and Inuyama, Y. (1992). Clinical significance of the epidermal growth factor receptor gene in squamous cell carcinomas of the nasal cavities and paranasal sinuses. *Cancer* 69, 358-362.

Gerwin, B.I. (1992). Overexpression of the $p185^{erbB-2}$ tyrosine kinase growth factor receptor: Control or chaos. *American Journal of Respiratory Cell Molecular Biology* 6, 357-358.

Gladhaug, I.P., Refsnes, M. and Christoffersen, T. (1992). Regulation of surface expression of high-affinity receptors for epidermal growth factor (EGF) in hepatocytes by hormones, differentiating agents, and phorbol ester. *Digestive Diseases and Sciences* 37, 233-239.

Goodyer, P.R., Fata, J., Mulligan, L., Fischer, D., Fagan, R., Guyda, H.J. and Goodyer, C.J. (1991). Expression of transforming growth factor α and epidermal growth factor receptor in human foetal kidneys. *Molecular and Cellular Endocrinology* 77, 199-206.

Gope, M., Gope, R. and Zarucki, T. (1991). Effect of dexamethasone and cycloheximide on the expression of amplified EGF-receptor gene in human A431 carcinoma cell line. *Molecular and Cellular Biochemistry* 103, 149-154.

Goustin, A.S., Leof, E.B., Shipley, G.D. and Moses, H.L. (1986). Growth factors and cancer. *Cancer Research* 46, 1015-1029.

Gullick, W.J. (1991). Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. *British Medical Bulletin* 47, 87-98.

Haffejee, A.A. and Bryer, J.V. (1991). Squamous cell oesophageal carcinoma - A review. *Gastroenterology Forum* 2, 23-37.

Hanauske, A.R., Buchok, J., Scheithauer, W. and Von Hoff, D.D. (1987). Human colon cancer cells secrete α TGF-like activity. *British*

Journal of Cancer 55, 57-59.

Heiss, N.S. (1992). The *ras*, *myc*, *fos* and *jun* oncogenes in human oesophageal cancer. MSc dissertation, University of the Witwatersrand, Johannesburg.

Heldin, C-H. and Westermark, B. (1989). Growth factors as transforming proteins. *European Journal of Biochemistry* 184, 487-496.

Helin, K. and Beguinot, L. (1991). Internalisation and down-regulation of the human epidermal growth factor receptor by the carboxyl-terminal tyrosines. *The Journal of Biological Chemistry* 266, 8363-8368.

Hofer, D.R., Sherwood, E.R., Bromberg, W.D., Mendelsohn, J., Lee, C. and Kozlowski, J.M. (1991). Autonomous growth of androgen-independent human prostatic carcinoma cells: role of transforming growth factor α. *Cancer Research* 51, 2780-2785.

Hollstein, M.C., Smits, A.M., Galiana, C., Yamasaki, H., Bos, J.L., Mandard, A., Partensky, C. and Montesano, R. (1988). Amplification of epidermal growth factor receptor gene but no evidence of *ras* mutations in primary human oesophageal cancers. *Cancer Research* 48, 5119-5123.

Honge, M., Itoi, M., Yamaguchi, N. and Imanishi, J. (1992). Distribution of epidermal growth factor (EGF) receptors in rabbit corneal epithelial cells, keratocytes and endothelial cells, and the changes induced by transforming growth factor-$ß_1$. *Experimental Eye Research* 54, 9-16.

Humphrey, P.A., Gangarosa, L.M., Wong, A.J., Archer, G.E., Lund-Johansen, M., Bjerkvig, R., Laerum, O-D., Friedman, H.S., Binger, D.D. (1991). Deletion-mutant epidermal growth factor receptor in human gliomas: Effect of type II mutation on receptor function. *Biochemical and Biophysical Research Communications* 178, 1413-1420.

Hunter, T. (1991). Co-operation between oncogenes. *Cell* 64, 249-270.

Hurtt, M.R., Moossy, J., Donovan-Peluso, M. and Locker, J. (1992). Amplification of epidermal growth factor receptor gene in gliomas: histopathology and prognosis. *The Journal of Neuropathology and Experimental Neurology* 51, 84-90.

Ibbotson, K.J., Harrod, J., Gowen, M., D'Souza, S., Smith, D.D., Winkler, M.E., Derynck, R. and Mundy, G. (1986). Human recombinant transforming growth factor α stimulates bone resorbtion and inhibits formation *in vitro*. *Proceedings of the National Academy of Sciences of the U.S.A.* 83, 2228-2232.

Ihara, S., Maeda-Takekoshi, F., Takekoshi, M., Yokoyama, M., Sakuma, S. and Watanabe, Y. (1991). Evidence that the tyrosine kinase domain of a small fraction of epidermal growth factor receptor molecules is exposed on the outer surface of A431 cells. *Cell Structure and Function* 16, 217-223.

Jones, G.J. (1989). EGF-induced cellular migratory and cytoskeletal responses. BSc (Honours) minor dissertation, University of the Witwatersrand, Johannesburg.

Jones, G.J., Heiss, N.S., Veale, R.B. and Thornley, A.L. (1992).

Amplification and expression of the TGF-α, EGF receptor and c-*myc* genes in four human oesophageal squamous cell carcinoma cell lines. *South African Journal of Science*, to be submitted.

Kaplan, P.L., Anderson, M and Ozanne, B. (1982). Transforming growth factor(s) production enables cells to grow in the absence of serum: an autocrine system. *Proceedings of the National Academy of Sciences of the U.S.A.* 79, 485-489.

Karnes, W.E., Walsh, J.H., Wu, V.S., Kim, R.S., Martin, M.G., Wong, H.C., Mendelsohn, J., Park, J-G. and Cuttitta, F. (1992). Autonomous proliferation of colon cancer cells that coexpress transforming growth factor α and its receptor. *Gastroenterology* 102, 474-485.

Kawamoto. T., Takahashi, K., Nishi, M., Kimura, T., Matsumura, T. and Taniguchi, S. (1991). Quantitative assays of epidermal growth factor receptor in human squamous cell carcinomas of the oral region by an avidin-biotin method. *Japanese Journal of Cancer* 82, 403-410.

Kearsley, J.H., Leonard, J.H., Walsh, M.D. and Wright, G.R. (1991). A comparison of epidermal growth factor receptor (EGF-R) and c-*erb*B-2 oncogene expression in head and neck squamous cell carcinomas. *Pathology* 23, 189-194.

Kim, I., Manni, A., Lynch, J. and Demers, L. (1991). Polyamine involvement in the secretion and action of TGF-α in hormone sensitive human breast cancer cells in culture. *Breast Cancer Research and Treatment* 18, 83-91.

Klein, S.B., Fisher, G.J., Jensen, T.C., Mendelsohn, J., Voorhees, J.J. and Elder, J.T. (1992). Regulation of TGF-α expression in human keratinocytes: PKC-dependent and independent pathways. *Journal of Cellular Physiology* 151, 326-336.

Koland, J.G. and Cerione, R.A. (1988). Growth factor control of epidermal growth factor receptor kinase activity via an intramolecular mechanism. *The Journal of Biological Chemistry* 263, 2230-2237.

Korc, M., Chandrasekar, B. and Shah, G.N. (1991). Differential binding and biological activities of epidermal growth factor and transforming growth factor α in a human pancreatic cancer cell line. *Cancer Research* 51, 6243-6249.

Kudlow, J.E., Leung, A.W.C., Korbin, M.S., Paterson, A.J. and Asa, S. (1989). Transforming growth factor-α in the mammalian brain. *The Journal of Biological Chemistry* 264, 3880-3883.

Kumar, R. and Mendelsohn, J. (1990). Growth regulation of A431 cells. *The Journal of Biological Chemistry* 265, 4578-4582.

Kurachi, H., Morishige, K-i., Amemiya, K., Adachi, H., Hirota, K., Miyake, A. and Tanizawa, O. (1991). Importance of transforming growth factor α/epidermal growth factor receptor autocrine growth mechanism in an ovarian cancer cell line *in vivo*. *Cancer Research* 51, 5956-5959.

Kuwano, H., Baba, K., Ikebe, M., Kitamura, K., Adachi, Y., Mori, M. and Sugimachi, K. (1992). Gastric involvement of oesophageal squamous cell carcinoma. *The British Journal of Surgery* 79, 328-

330.

Kwatra, M.M., Bigner, D.D. and Cohn, J.A. (1992). The ligand binding domain of the epidermal growth factor receptor is not required for receptor dimerisation. *Biochemica et Biophysica Acta* 1134, 178-181.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lazar, L.M. and Blum, M. (1992). Regional distribution and developmental expression of epidermal growth factor and transforming growth factor-α mRNA in mouse brain by a quantitative nuclease protection assay. *The Journal of Neuroscience* 12, 1688-1697.

Lee, D.C., Rose, T.M., Webb, N.R. and Todaro, G.J. (1985). Cloning and sequence analysis of a cDNA for rat transforming growth factor-α. *Nature* 313, 489-491.

Lemoine, N.R., Hughes, C.M., Barton, C.M., Poulsom, R., Jeffery, R.E., Klöppel, G., Hall P.A. and Gullick, W.J. (1992). The epidermal growth factor receptor in human pancreatic cancer. *The Journal of Pathology* 166, 7-12.

Lewis, J.J., Goldenring, J.R., Modlin, I.M. and Coffey, R.J. (1990). Inhibition of parietal cell H^+ secretion by transforming growth factor alpha: A possible autocrine regulatory mechanism. *Surgery* 108, 220-227.

Lin, C.R., Chen, W.S., Kruiger, W., Stolarsky, L.S., Weber, W., Evans,

R.M., Verma, I.M., Gill, G.N. and Rosenfeld, M.G. (1984). Expression cloning of human EGF receptor complementary DNA: Gene amplification and three related messenger RNA products in A431 cells. *Science* 224, 843-848.

Liu, X. and Pawson, T. (1991). The epidermal growth factor receptor phosphorylates GTPase-activating protein (GAP) at Tyr-460, adjacent to the GAP SH2 domains. *Molecular and Cellular Biology* 11, 2511-2516.

Lu, S-H., Hsieh, [illegible], Luo, F-C. and Weinstein, I.B. (1988). Amplification of the EGF receptor and *c-myc* genes in human oesophageal cancers. *International Journal of Cancer* 42, 502-505.

Luetteke, N.C., Michalopoulos, G.K., Teixidó, J., Gilmore, R., Massagué, J. and Lee, D.C. (1988). Characterisation of high molecular weight transforming growth factor α produced by rat hepatocellular carcinoma cells. *Biochemistry* 27, 6487-6494.

Lyons, R.M. and Moses, H.L. (1990). Transforming growth factors and the regulation of cell proliferation. *European Journal of Biochemistry* 187, 467-473.

Madtes, D.K., Raines, E.W., Sakariassen, K.S., Assoian, R.K., Sporn, M.B., Bell, G.I. and Russell, R. (1988). Induction of transforming growth factor-α in activated human alveolar macrophages. *Cell* 53, 285-293.

Magni, M., Pandiella, A., Helin, K., Meldolesi, J. and Beguinot, L. (1991). Transmembrane signalling at the epidermal growth factor receptor. *Journal of Biochemistry* 277, 305-311.

Markowitz, S.D., Molkentin, K., Gerbic, C., Jackson, J., Stellato, T. and Willson, J.K.V. (1990). Growth stimulation by coexpression of transforming growth factor-α and epidermal growth factor receptor in normal and adenomatous human colon epithelium. *The Journal of Clinical Investigation* 86, 356-362.

Maruo, T., Yamasaki, M., Ladines-Llave, C.A. and Mochizuki, M. (1992). Immunohistochemical demonstration of elevated expression of epidermal growth factor receptor in the neoplastic changes of cervical squamous epithelium. *Cancer* 69, 1182-1187.

Massagué, J. (1983). Epidermal growth factor-like transforming growth factor. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. *The Journal of Biological Chemistry* 258, 13614-13620.

Massagué, J. (1985). Transforming growth factors. Isolation, characterisation and interaction with cellular receptors. *Progress in Medical Virology* 32, 142-158.

Massagué, J. (1990). Transforming growth factor-α. A model for membrane-anchored growth factors. *The Journal of Biological Chemistry* 265, 21393-21396.

Masui, H., Wells, A., Lazar, C.S., Rosenfeld, M.G. and Gill, G.N. (1991). Enhanced tumourigenesis of NR6 cells which express non-down-regulating epidermal growth factor receptors. *Cancer Research* 51, 6170-6175.

Mead, J.E. and Fausto, N. (1989). Transforming growth factor α may be a physiological regulator of liver regeneration by means of an

autocrine mechanism. *Proceedings of the National Academy of Sciences of the U.S.A.* 86, 1558-1562.

Meisenhelder, J., Suh, P-G., Rhee, S.G. and Hunter, T. (1989). Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases *in vivo* and *in vitro*. *Cell* 57, 1109-1122.

Mercola, M. and Stiles, C.D. (1988). Growth factor superfamilies and mammalian embryogenesis. *Development* 102, 451-460.

Merlino, G.T., Xu, Y-H., Ishii, S., Clark, A.J.L., Semba, K., Toyoshima, K., Yamamoto, T. and Pastan, I. (1984). Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. *Science* 224, 417-419.

Miettinen, P.J. and Heikinheimo, K. (1992). Transforming growth factor-alpha (TGF-α) and insulin gene expression in human foetal pancreas. *Development* 114, 833-840.

Miller, S.A., Dykes, D.D. and Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* 16, 1215.

Mooibroek, M.J., Cheng, H-C. and Wang J.H. (1992). Differential *in vitro* phosphorylation of clathrin light chains by the epidermal growth factor receptor-associated protein tyrosine kinase and a $pp60^{c\text{-}src}$ related spleen tyrosine kinase. *Archives of Biochemistry and Biophysics* 292, 448-455.

Morishige, K-i., Kurachi, H., Amemiya, K., Adachi, H., Inoue, M., Miyake, A., Tanizawa, O. and Sakoyama, Y. (1991). Involvement

of transforming growth factor α/epidermal growth factor receptor autocrine mechanism in an ovarian cancer cell line *in vitro*. *Cancer Research* 51, 5951-5955.

Munson, P.J. and Rodbard, D. (1980). A versatile computerised approach for characterisation of ligand-binding systems. *Analytical Biochemistry* 107, 220-239.

Myrdal, S.E., Twardzik, D.R. and Auersperg, N. (1986). Cell-mediated co-action of transforming growth factors: incubation of type β with normal rat kidney cells produces a soluble activity that prolongs the ruffling response to type α. *The Journal of Cell Biology* 102, 1230-1234.

Nair, N., Davis, K.J. and Robinson, H.L. (1992). Protein tyrosine kinase activities of the epidermal growth factor receptor and erbB proteins: Correlation of oncogenic activation with altered kinetics. *Molecular and Cellular Biology* 12, 2010-2016.

Nickoloff, B.J., Mitra, R.S., Riser, B.L., Dixit, V.M. and Varani, J. (1988). Modulation of keratinocyte motility: Correlation with the production of extracellular molecules in response to growth promoting and anti-proliferative factors. *The American Journal of Pathology* 132, 543-551.

Nielsen L.L., Werb, Z. and Pedersen, R.A. (1991). Induction of c-*fos* transcripts in early postimplantation mouse embryos by TGF-α, EGF, PDGF, and FGF. *Molecular Reproduction and Development* 29, 227-237.

Ohlsson, R.I. and Pfeifer-Ohlsson, S.B. (1987). Cancer genes, proto-

oncogenes, and development. *Experimental Cell Research* 173, 1-16.

Ojeda, S.R., Urbanski, H.F., Costa, M.E., Hill, D.F. and Moholt-Siebert, M. (1990). Involvement of transforming growth factor α in the release of luteinizing hormone-releasing hormone from the developing female hypothalamus. *Proceedings of the National Academy of Sciences of the U.S.A.* 87, 9698-9702.

Ozanne, B., Richards, C.S., Hendler, F., Burns, D. and Gusterson, B. (1986). Overexpression of the EGF receptor is a hallmark of squamous cell carcinomas. *The Journal of Pathology* 149, 9-14.

Ozawa, S., Ueda, M., Ando, N., Abe, O. and Shimizu, N. (1987). High incidence of EGF receptor hyperproduction in oesophageal squamous-cell carcinomas. *International Journal of Cancer* 39, 333-337.

Ozawa, S., Ueda, M., Ando, N., Shimizu, N. and Abe, O. (1989). Prognostic significance of epidermal growth factor receptor in oesophageal squamous cell carcinomas. *Cancer* 63, 2169-2173.

Pandiella, A. and Massagué, J. (1991a). Cleavage of the membrane precursor for transforming growth factor α is a regulated process. *Proceedings of the National Academy of Sciences of the U.S.A.* 88, 1727-1730.

Pandiella, A. and Massagué, J. (1991b). Multiple signals activate cleavage of the membrane transforming growth factor-α precursor. *The Journal of Biological Chemistry* 266, 5769-5773.

Pandiella, A. and Massagué, J. (1991c). Transforming growth factor-α. *Biochemical Society Transactions* 19, 259-262.

Pittelkow, M.R., Lindquist, P.B., Abraham, R.T., Graves-Deal, R., Derynck, R. and Coffey, R.J. (1989). Induction of transforming growth factor-α expression in human keratinocytes by phorbol esters. *The Journal of Biological Chemistry* 264, 5164-5171.

Pollak, M., Boyarsky, A. and Gora, P. (1991). A mathematical model describing consequences of abnormally high levels of epidermal growth factor receptor on the proliferation of neoplastic cells. *Cancer Investigation* 9, 513-520.

Qian, X., Decker, S.J. and Greene, M.I. (1992). $p185^{c\text{-}neu}$ and epidermal growth factor receptor associate into a structure composed of activated kinases. *Proceedings of the National Academy of Sciences of the U.S.A.* 89, 1330-1334.

Ramsdell, J.S. (1991). Transforming growth factor-α and -β are potent and effective inhibitors of GH_4 pituitary tumour cell proliferation. *Endocrinology* 128, 1981-1990.

Rappolee, D.A., Mark, D., Banda, M.J. and Werb, Z. (1988). Wound macrophages express TGF-α and other growth factors *in vivo*: analysis by mRNA phenotyping. *Science* 241, 708-712.

Redemann, N., Holzmann, B., von Rüden, T., Wagner, E.F., Schlessinger, J. and Ullrich, A. (1992). Anti-oncogenic activity of signalling-defective epidermal growth factor receptor mutants. *Molecular and Cellular Biology* 12, 491-498.

Reiss, M., Stash, E.B., Vellucci, V.F. and Zhou, Z-L. (1991). Activation of the autocrine transforming growth factor α pathway in human squamous carcinoma cells. *Cancer Research* 51, 6254-6262.

Riedel, H., Massoglia, S., Schlessinger, J. and Ullrich, A. (1988). Ligand activation of overexpressed epidermal growth factor receptor transforms NIH 3T3 mouse fibroblasts. *Proceedings of the National Academy of Sciences of the U.S.A.* 85, 1477-1481.

Rosenthal, A., Lindquist, P.B., Bringman, T.S., Goeddel, D.V. and Derynck, R. (1986). Expression in rat fibroblasts of a human transforming growth factor-α cDNA results in transformation. *Cell* 46, 301-309.

Rotin, D., Honneger, A.H., Margolis, B.L., Ullrich, A. and Schlessinger, J. (1992). Presence of SH2 domains of phospholipase $C\gamma_1$ enhances substrate phosphorylation by increasing the affinity toward the epidermal growth factor receptor. *The Journal of Biological Chemistry* 267, 9678-9683.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual. Second edition (New York: Cold Spring Harbour Laboratory Press), pp 1.25-1.28.

Samsoondar, J., Kobrin, M.S. and Kudlow, J.E. (1986). α-Transforming growth factor secreted by untransformed bovine anterior pituitary cells in culture. *The Journal of Biological Chemistry* 261, 14408-14413.

Schlessinger, J. (1988). Signal transduction by allosteric receptor oligomerisation. *Trends in Biochemical Sciences* 13, 443-447.

Schreiber, A.B., Winkler, M.E. and Derynck, R. (1986). Transforming growth factor α: a more potent angiogenic mediator than epidermal growth factor. *Science* 232, 1250-1253.

Schultz, G., Rotatori, D.S. and Clark, W. (1991). EGF and TGF-α in wound healing and repair. *Journal of Cellular Biochemistry* 45, 346-352.

Seroogy, K.B., Han, V.K.M. and Lee, D.C. (1991). Regional expression of transforming growth factor-α mRNA in the rat central nervous system. *Neuroscience Letters* 125, 241-245.

Siegel, S. (1956). Nonparametric statistics for the behavioural sciences (Tokyo: McGraw-Hill Kogakusha Ltd.), pp 116-127.

Singletary, S.E., Williams, N.N., Rodeck, U., Larry, L., Tucker, S., Spitzer, G. and Herlyn, M. (1990). Transforming growth factor-alpha secretion by epidermal growth factor-dependent human tumour cell lines. *Anticancer Research* 10, 1501-1506.

Smith, J.J., Derynck, R. and Korc, M. (1987). Production of transforming growth factor α in human pancreatic cancer cells: Evidence for a superagonist autocrine cycle. *Proceedings of the National Academy of Sciences of the U.S.A.* 84, 7567-7570.

Solomon, E., Borrow, J. and Goddard, A.D. (1991). Chromosome aberrations and cancer. *Science* 254, 1153-1160.

Sorkin, A. and Carpenter, G. (1991). Dimerisation of internalised epidermal growth factor receptors. *The Journal of Cell Biology* 266, 23453-23460.

Sorkin, A., Waters, C., Overholser, K.A., and Carpenter, G. (1991). Multiple autophosphorylation site mutations of the epidermal growth factor receptor. *The Journal of Biological Chemistry* 266, 8355-8362.

Sporn, M.B. and Roberts, A.B. (1991). Autocrine growth factors and the regulation of tumour growth. In Molecular foundations of oncology, S. Broder, ed. (Baltimore: Williams and Wilkins), pp 393-402.

Stark, G.R., Debatisse, M., Giulotto, E. and Wahl, G.M. (1989). Recent progress in understanding mechanisms of mammalian DNA amplification. *Cell* 57, 901-908.

Stromberg, K., Collins, T.J., Gordon, A.W., Jackson, C.L. and Johnson, G.R. (1992). Transforming growth factor-α acts as an autocrine growth factor in ovarian carcinoma cell lines. *Cancer Research* 52, 341-347.

Tam, J.P., Lin, Y-Z., Liu, W., Wang, D-X., Ke, X-H. and Zhang, J-W. (1991). Mapping the receptor-recognition site of human transforming growth factor-α. *International Journal of Protein Research* 38, 204-211.

Tamada, H., Das, S.K., Andrews, G.K. and Dey, S.K. (1991). Cell-type-specific expression of transforming growth factor-α in the mouse uterus during the peri-implantation period. *Biology of Reproduction* 45, 365-372.

Teixidó, J., Gilmore, R., Lee, D.C. and Massagué, J. (1987). Integral membrane properties of the prohormone pro-transforming growth

factor-α. *Nature* 326, 883-885.

Teixidó, J. and Massagué, J. (1988). Structural properties of a soluble bioactive precursor for transforming growth factor-α. *The Journal of Biological Chemistry* 263, 3924-3929.

Teixidó, J., Wong, S.T., Lee, D.C. and Massagué, J. (1990). Generation of transforming growth factor-α from the cell surface by an O-glycosylation-independent multi-step process. *The Journal of Biological Chemistry* 265, 6410-6415.

Theodorescu, D., Cornil, I., Sheehan, C., Man, M.S. and Kerbel, R.S. (1991). Ha-*ras* induction of the invasive phenotype results in up-regulation of epidermal growth factor receptors and altered responsiveness to epidermal growth factor in human papillary transitional cell carcinoma cells. *Cancer Research* 51, 4486-4491.

Thornley, A.L. and Jones, G.J. (1992). *In vitro* secretion of transforming growth factor alpha (TGF-α): A comparison of the A431 cell line with three human oesophageal squamous cell carcinoma cell lines. *Bioscience Reports* 12, 293-302.

Thornley, A.L., Scott, E., Manchester, K.L. and Veale, R.B. (1991). Human oesophageal squamous carcinoma cells: histological features and DNA profiling of six cell lines. *British Journal of Cancer*, submitted.

Todaro, G.J., Fryling, C. and De Larco, J. (1980). Transforming growth factors produced by certain human tumour cells: Polypeptides that interact with epidermal growth factor receptors. *Proceedings of the National Academy of Sciences of the U.S.A.* 77, 5258-5262.

Todd, R., Donoff, B.R., Chiang, T., Chou, M.Y., Elovic, A., Gallagher, G.T. and Wong, D.T.W. (1991). The eosinophil as a cellular source of transforming growth factor alpha in healing cutaneous wounds. *American Journal of Pathology* 138, 1307-1313.

Torp, S.H., Helseth, E., Dalen, A. and Unsgaard, G. (1991). Epidermal growth factor receptor expression in human gliomas. *Cancer Immunology and Immunotherapy* 33, 61-64.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the U.S.A.* 76, 4350-4354.

Turksen, K., Choi, Y. and Fuchs, E. (1991). Transforming growth factor alpha induces collagen degradation and cell migration in differentiating human epidermal raft cultures. *Cell Regulation* 2, 613-625.

Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D. and Seeburg, P.H. (1984). Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309, 418-425.

Ullrich, A. and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* 61, 203-212.

van Belzen, N., Rijken, P.J., Verkleij, A.J. and Boonstra, J. (1991). Sulphydryl reagents alter epidermal growth factor receptor

affinity and association with the cytoskeleton. *Journal of Receptor Research* 11, 919-940.

van Bergen en Henegouwen, P.M.P., den Hartigh, J.C., Romeyn, P., Verkleij, A.J. and Boonstra, J. (1992). The epidermal growth factor receptor is associated with actin filaments. *Experimental Cell Research* 199, 90-97.

Van de Vijver, M.J., Kumar, R. and Mendelsohn, J. (1991). Ligand-induced activation of A431 cell epidermal growth factor receptors occurs primarily by an autocrine pathway that acts upon receptors on the surface rather than intracellularly. *The Journal of Biological Chemistry* 266, 7503-7508.

Veale, R.B. and Thornley, A.L. (1989). Increased single class low-affinity EGF receptors expressed by human oesophageal squamous carcinoma cell lines. *South African Journal of Science* 85, 375-379.

Vega, Q.C., Cochet, C., Filhof, O., Chang, C-P., Rhee, S.G. and Gill, G.N. (1992). A site of tyrosine phosphorylation in the C terminus of the epidermal growth factor receptor is required to activate phospholipase C. *Molecular and Cellular Biology* 12, 128-135.

Velu, T.J. (1990). Structure, function and transforming potential of the epidermal growth factor receptor. *Molecular and Cellular Endocrinology* 70, 205-216.

Walton, G.M., Chen, W.S., Rosenfeld, M.G. and Gill, G.N. (1990). Analysis of deletions of the carboxyl terminus of the epidermal growth factor receptor reveals self-phosphorylation at tyrosine 992

and enhanced *in vivo* tyrosine phosphorylation of cell substrates. *The Journal of Biological Chemistry* 265, 1750-1754.

Wang, S-L., Jacober, L., Wu-Wang, C-Y., Slomiany, A. and Slomiany, B.L. (1992). Ethanol-induced structural and functional alteration of epidermal growth factor receptor in buccal mucosa. *International Journal of Biochemistry* 24, 85-90.

Wedegaertner, P.B. and Gill, G.N. (1992). Effect of carboxyl terminal truncation on the tyrosine kinase activity of the epidermal growth factor receptor. *Archives of Biochemistry and Biophysics* 292, 273-280.

Weinberg, R.A. (1991a). Molecular biology of carcinogenesis: A multi-step process. In Molecular foundations of oncology, S. Broder, ed. (Baltimore: Williams and Wilkins), pp 27-39.

Weinberg, R.A. (1991b). Tumour suppressor genes. *Science* 254, 1138-1146.

Wong, D.T.W., Weller, P.F., Galli, S.J., Elovic, A., Rand, T.H., Gallagher, G.T., Chiang, T., Chou, M.Y., Matossain, K., McBride, J., and Todd, R. (1990). Human eosinophils express transforming growth factor α. *The Journal of Experimental Medicine* 172, 673-681.

Wong, S., Winchell, L.F., McCune, B.K., Shelton Earp, H., Teixidó, J., Massagué, J., Herman, B. and Lee, D.C. (1989). The TGF-α precursor expressed on the cell surface binds to the EGF-receptor on adjacent cells, leading to signal transduction. *Cell* 56, 496-506.

Wood, D.P., Fair, W.R. and Chaganti, R.S.K. (1992). Evaluation of epidermal growth factor receptor DNA amplification and mRNA expression in bladder cancer. *The Journal of Urology* 147, 274-277.

Xu, Y.H., Richert, N., Ito, S., Merlino, G.T. and Pastan, I. (1984). Characterisation of epidermal growth factor receptor gene expression in malignant and normal human cell lines. *Proceedings of the National Academy of Sciences of the U.S.A.* 81, 7308-7312.

Yamamoto, T., Kamata, N., Kawano, H., Shimizu, S., Kuroki, T., Toyishima, K., Rikimaru, K., Nomura, N., Ishizaki, R., Pastan, I., Gamou, S. and Shimizu, N. (1986). High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. *Cancer Research* 46, 414-416.

Yoshida, K., Kyo, E., Tsuda, T., Tsujino, T., Ito, M., Niioto, M. and Tahara, E. (1990). EGF and TGF-α, the ligands of hyperproduced EGF-R in human oesophageal carcinoma cells, act as autocrine growth factors. *International Journal of Cancer* 45, 131-135.