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# The Effects of Growth Factors on Proliferation, Survival and Motility in a Human Embryonal Carcinoma Derived Cell Line

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SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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**Abstract**

The balance between different cell populations in the developing organism is controlled by regulating the rates of multiplication, differentiation or death of their constituent cells. In the absence of an human embryonic stem cell that can be studied in vitro, the embryonal carcinoma derived cell line Tera-2 has for long been considered the second best alternative for studies in vitro. This cell line is multipotent in vivo and can be induced to undergo differentiation in vitro by addition of retinoic acid.

In this thesis, it is reported that differentiation has a marked effect on the expression of one growth factor gene in vitro. K-FGF mRNA was rapidly downregulated by addition of retinoic acid. When cells were cultured in RA for an extended period of time (> 15 days) the K-FGF transcript reappeared.

When Tera-2 cells were grown in serum-free medium, the increase in total cell number was stimulated by the addition of 1-10 ng/ml medium of aFGF or bFGF. The FGF effect was specific and could be abrogated by protamine sulphate. At least two classes of FGF receptors were found to bind to FGF in Tera-2 cells. By a combination of cell counts, autoradiography and immunohistochemistry it was possible to conclude that Tera-2 cells, grown in serum-free medium undergo apoptosis.

The stimulatory effects of aFGF and bFGF on cell numbers could be concluded to be the result of the growth factors abrogating the apoptotic process. By adding the Insulin-like growth factor I or II to Tera-2 cells in serum-free medium an even greater survival effect was observed.

Basic FGF at high concentrations exerted only a marginal effect on cell proliferation, but had a preferential effect on cell locomotion, supporting the concept that one growth factor can give rise to different biological effects at different concentrations.

*Key words*: Growth factors, Embryonal Carcinoma, cell proliferation, cell motility, apoptosis.

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# Appendix

## Papers I-V

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals I-V.

I. Schofield, P.N., Ekström, T.J., Granerus, M. and Engström, W. (1991). Differentiation associated modulation of K-FGF expression in a human teratocarcinoma cell line and in primary germ cell tumours. *FEBS letters* **280**; 8-10.

II. Schofield, P.N., Granerus, M., Lee, A., Ekström, T.J. and Engström, W. (1992). Concentration dependent modulation of fibroblast growth factor action on multiplication and locomotion of human teratocarcinoma cells. *FEBS letters* **298**; 154-156.

III. Granerus, M., Bierke, P., Zumkeller, W., Smith, J. and Engström, W. (1995). Insulin-like growth factor II prevents apoptosis in a human teratoma cell line. *J. Clin. Pathology* **48**; M 153-157.

IV. Granerus, M., Schofield, P.N., Bierke, P. and Engström, W. (1995). The role of IGF I and TGFβ I in regulating cell growth and cell death in a human teratocarcinoma derived cell line. *Int J Devl Biology* **39**; 759-764.

V. Granerus, M .and Engström, W. (1996). Growth factors and apoptosis. *Cell proliferation* **29**; 309-314.

# Aims of the thesis

The aims of this thesis were

- To further characterize the growth phenotype of the human embryonal carcinoma derived cell line Tera-2.

- To examine how members of the heparin binding growth factor family and the insulin-like growth factor family elicit different biological responces in this cell line.

- To elucidate how growth factors affect the balance between survival and death in this cell line.

# Contents

# Introduction

## Human germ cell tumours

*Clinical features of human germ cell tumours*

Germ cell tumours represent a broad range of neoplasms which develop in the gonads and extragonadal sites. Germ cell tumours can also arise as primary tumours in extragonadal sites. Usually these tumours are found in the mediastinum, the retroperitoneum, the sacrococcygeal region, or in the III ventricle of the brain (Munro, 1986). Even though teratomas occasionally in the ovary, the main interest has been focused on germ cell tumours arising in the testis. Even if testicular tumours are relatively rare, with an incidence rate of 3 per 100.000 in western Europe and the United States, they mainly develop in men between 20 and 35. In this group it is the most common neoplasm (Waterhouse, 1986, Skakkebaeck, 1978).

Clinical and experimental evidence suggests that initiation of testicular germ cell tumours occurs in early fetal life. The vast majority of these tumours originate from germ line cells. Such cells are called primitive germ cells and resemble primordial germ cells which have completed the migration from the hindgut to the genital ridge in the early human embryo. Most human testicular tumours have been found to be aneuploid and often contain both X and Y chromosomes. Since the same pairs of alleles are present in the non-neoplastic cells of the patients body, it has been concluded that teratomas develop before the meiotic division of the germ cells (Graham, 1982).

Testicular germ cell tumours can be divided into two categories; tumours which consist of one histological type, and those of more than one histological type. Tumours with a monotypic histological pattern comprise 60% of all testicular neoplasms and include seminoma, embryonal carcinoma, choriocarcinoma and yolk sac tumour. The remaining 40% of the tumours contain a mixture of two or more of these five histological patterns. The most common contain elements of teratoma and embryonal carcinoma, and are therefore usually referred to as teratocarcinoma. All of these tumours arise in testicular germ cells. The germ cell could give rise to a seminoma representing gonadal differentiation, or transform into a pluripotent tumour cell representing embryonal carcinoma, which is the stem cell for all non-seminomatous germ cell tumours. Depending on the degree of differentiation of embryonal carcinoma cells, tumours with different histological patterns will develop. Pure embryonal carcinoma contains undifferentiated stem cells only, yolk sac carcinoma and choriocarcinoma contain tumour cells with features of extra-embryonic endoderm and trophectoderm respectively. Teratoma results from differentiation of the embryonic carcinoma cells into derivatives of the three germ cell layers. Many of these tumours are believed to be

derived from cell types identified as carcinoma in situ, a possibly pre-neoplastic state from which cells may progress and accumulate karyotypic changes.

*Germ cell tumours and embryogenesis*

Early experimental studies revealed that there exists a close relationship between normal embryogenesis and development of embryonic neoplasms. The classic experiments of Leroy Stevens showed that mice of certain inbred strains frequently develop tumours analogous to human teratocarcinomas (Stevens, 1970). As cell lines were established from these murine tumours, it became obvious that the cultured cells - known as embryonal carcinoma (EC) cells or teratocarcinoma stem cells - were remarkably similar to early embryonic cells. Moreover, early post-implantation embryos grafted to extra-uterine sites were found to develop into teratoma or teratocarcinomas. Again, cell lines from experimentally induced teratocarcinomas were established in vitro and were found to have retained some of their developmental potential. Early examples of such murine embryonal carcinoma (EC) cells were the F9, PC13 (Bernstine et al., 1973), P 19 (McBurney and Rogers, 1982) and the PSA-1 lines (Martin et al., 1977). Some of these cells showed phenotypic characteristics and developmental potential equivalent to the inner cell mass or the embryonic ectoderm of the early post-implantation embryo. In an experiment that lasted over an eight year period, Illmensee and Mintz, 1975 took 6 day mouse embryos and placed them under the testis capsule where they formed teratocarcinomas which metastasized to the renal lymph node. The primary tumour was minced and transplanted intraperitoneally where it formed an ascitic tumour of embryoid bodies which consisted of yolk sac rinds and embryonal carcinoma cores. Core EC cells were injected into blastocysts and in spite of the demonstrable ability to generate transplantable malignant tumours, live mice were born. In a subsequent experiment Illmensee and Mintz injected EC cells into the blastocoelic cavity of normal embryos. When the chimaeric embryos were reintroduced into foster mothers, the EC-cells contributed to a wide range of chimaeric tissues in the off-spring.

These early experiments did much to promote our understanding of the close relationship between normal embryogenesis and the development of germ cell tumours. Two obvious conclusions immediately followed. First: when early embryonic cells are removed from their normal environment it leads to malignant behaviour of these cells. Secondly, the transformed phenotype is clearly reversible, since malignant EC cells derived from the embryo induced teratocarcinomas revert to a normal behaviour when reintroduced in the normal embryonic environment. When this context was clarified, developmental biologists began an intense characterization of the growth phenotype of EC cells, in the general hope that these studies would yield clues to how embryonic differentiation is regulated.

*Mouse embryonal carcinoma*

Developmental biologists have for decades proposed the existence of a mutual growth promoting effect of embryonic and extraembryonic components of the mouse conceptus. In a classical experiment Richard Gardner demonstrated that the inner cell mass secreted diffusable factors capable of supporting growth co-operativity between the inner cell mass and the trophectoderm in the early mouse embryo (Gardner, 1972). A similar interrelationship was demonstrated in some teratocarcinoma cell types (Heath and Rees, 1985). Murine teratocarcinomas produced either spontaneously in the 129/J strain or by ectopic transplantation of early embryos, are composed of two components; a malignant stem cell and its differentiated progeny. A progressively growing component can be isolated from such tumours which may show more or less the characteristics of more or less differentiation (Damjanov et al., 1987). The progressively growing stem cells from the PC13 cell line share characteristics with primitive ectoderm. In vitro they can be induced to differentiate to give a population of primitive endoderm like cells (END-cells) with progressively lengthening intermitotic periods (Heath and Isacke, 1984). However, co-culture of mouse embryonal carcinoma cells with their differentiated progeny not only lead to enhanced survival of EC cells but also resulted in the induction of heterologous target cell DNA synthesis and cell multiplication (Isacke and Deller, 1983). It was proposed that one source of END cell growth promoting substances was the undifferentiated parent EC cells. The factor responsible for stimulating END cell multiplication by their EC cell progenitors was purified and termed Embryonal Carcinoma Derived Growth Factor (ECDGF), which was subsequently found to possess close biochemical properties to basic fibroblast growth factor (Heath and Isacke, 1984). The reciprocal part of this relationship seems to comprise the synthesis of IGF-II by the END cell population, which will stimulate the growth of the EC cells (Heath and Rees, 1985; Heath and Shi, 1986). These findings suggested that a potential growth reciprocity might exist between the stem cell population and their differentiated progeny in the mouse embryo. This concept was valuable in that it predicted that the relative sizes of the stem and differentiated cell compartments are decided by the rate of transition between them, and hence by the rate of differentiation (Heath and Rees, 1985). However, it remains to be shown that this indeed does happen in the embryo.

*Human embryonal carcinoma*

Two lines of evidence indicate that human EC cells are different from murine EC cells. Firstly, human EC cells express the closely related embryonic antigens SSEA-3 and SSEA-4, which are absent in murine EC cells (Kannagi et al., 1983; Shevinsky et al., 1982). In contrast, the distinct stage specific embryonic antigen SSEA-1 is expressed by murine, but not by human EC cells. In the murine embryo SSEA-3 and -4 are expressed by preimplantation embryos, while the SSEA-1 antigen is present at the surface of the inner cell mass cells and primitive ectoderm (Shevinsky et al., 1982; Solter and Knowles, 1978). Andrews then hypothe-

sized that the stem cells of human teratocarcinoma are related to cells of the preimplantation embryo (Andrew et al., 1987). Secondly, trophectodermal and other forms of extra-embryonic differentiation, in addition to somatic differentiation, are frequently observed in human teratocarcinoma (Damjanov, 1983). Trophectodermal elements are rare in murine teratocarcinoma (Pierce, 1983; Damjanov, 1985), indicating that the capacity to form trophectoderm which is present in human EC cells is lost in murine EC cells.

Since Jörgen Fögh's pioneering work (Fögh and Trempe, 1975) other groups have reported the establishment of cell lines from human testicular teratocarcinoma which were subsequently characterized and compared (Andrews, 1983). It was shown that the model human EC cell shows expression of high molecular weight protein, production of alkaline phosphatase, expression of HLA-A, B, C antigen, β-2-macroglobulin and expression of embryonic antigen SSEA-3 and 4, but not SSEA-1 (Andrews et al., 1983). The differential expression of the latter two antigens was confirmed in human teratocarcinoma specimen grown as xenografts in nude mice (Andrews et al., 1983). When examined by light-microscopy they histologically resemble murine EC-cells.

Most human EC cell lines are unable to undergo differentiation, either in vitro or when injected into nude mice (Andrews, 1980). Whether these cells are either genuinely nullipotent or just not exposed to the right differentiation induction protocol remains unclear. Nevertheless Pera et al. (1987), successfully established pluripotent cell lines from testicular germ cell tumours. Some of the cell lines gave rise to teratocarcinoma when injected into nude mice and are thus to be regarded as true multipotent cells.

## Growth factors

*Growth factors in embryogenesis*

Growth factors are multifunctional polypeptides that have a fundamental impact on the behaviour of almost every known cell type. They not only control cell growth and proliferation but also affect other basal cellular functions such as motility, differentiation and survival. Growth factors exert their biological effect by interacting with membrane receptors. Such receptors act as signal transducers and their key function is to relay a signal to the cell interior. To complete this function, the prototype receptor protein consists of an external ligand binding domain, a hydrophobic transmembrane stretch and an intracellular functional domain, which often possesses kinase activity.

The extent to which growth factors are made available to their target cells is dependent on their production and release, as well as tissue specificity. This concept is sometimes, but not always, implemented by fundamental differences in receptor phenotype. Moreover, the availability of growth factors is modulated by the

presence of specific binding proteins. Such molecules not only provide a transport mechanism but by virtue of their high affinity regulate the availability of growth factors.

It is becoming increasingly evident that growth factors play a central role in many embryonic processes. Not only are these molecules and their receptors present within the early embryo in vivo (Engström and Heath, 1988), but also initiate and modulate a variety of cellular events that are essential to the development of the organism. In *Xenopus blastulae* it has been demonstrated that growth factors initiate a process that commit a fraction of cells in isolated ectoderm to be differentiated into mesodermal cells (Slack, 1990). Much attention have recently been attributed to the regulatory elements of growth factor genes as well as to the transcription factors that affect the level of expression. In particular, interest has been focused on studying glucocorticoid receptor binding motifs (Dahlman-Wright, 1991), and the fos/jun protein interaction with AP-1 sites (Caricasole and Ward, 1993).

*The fibroblast growth factor family*

Fibroblast growth factor (FGF) was originally identified by Denis Gospodarowicz as a mitogen present in pituitary hormone and bovine brain. In his initial characterization Gospodarowicz found that FGF bound to immobilized heparin (a polysulphated glycosaminoglycan found in many tissues) and this heparin-binding capacity was considered to be a key feature for FGF (Gospodarowicz, 1978). The initial purification of FGF from calf brain revealed that the preparation contained two different molecules with similar biochemical and biological properties. They were denominated basic FGF (bFGF) and acidic FGF (aFGF) on the basis of their pI value (Gospodarowicz, 1984; Böhlen et al., 1985). Both aFGF and bFGF are powerful inducers of cell proliferation for a wide variety of cell types, including fibroblasts, neurectodermal cells and vascular endothelial cells. The transcriptional activity of the bFGF gene is extremely widespread in both adult and fetal tissues. Basic FGF is also expressed in a large number of normal and transformed cell lines. In contrast expression of aFGF is limited to cells of neural origin. Moreover, both aFGF and bFGF lack the hydrophobic signal sequence at their N-termini usually required for normal secretion from cells (Goldfarb, 1990). This has led several investigators to propose that aFGF and bFGF are not made available to responsive cells unless by mechanism that result from cell damage.

Since the original discovery seven additional members of the FGF family have been identified. The first of these, int-2 was identified as a gene located close to a frequent insertion site of the Mouse Mammary Tumour Virus (MMTV) in virally induced mammary tumours. The sequencing of the int-2 gene revealed a significant homology with the aFGF and bFGF genes. Further searches for related genes led to the discovery of K-FGF/hst (FGF-4) and FGF-5, FGF-6, FGF-8 and FGF-9. Another FGF-like growth factor, KGF (FGF-7) was discovered on the basis of its

mitogenic effects on keratinocyte-derived cell lines in culture. An important feature of all other FGF species is that they contain a functional secretory signal peptide and therefore can be exported from the cells.

Comparing the primary structure of the different FGF shows that the overall homology of the family is quite low, but that certain key residues are maintained in all nine cases. Moreover, these conserved residues seem to correspond to key structures in the 3 D-structure of bFGF.

*The insulin-like growth factors*

The existence of the insulin-like growth factors (IGF:s) or somatomedins was first predicted by Salmon and Daughaday (1957), who proposed that pituitary growth hormone exerted its effect on skeletal growth by using an intermediate class of growth promoting peptides. Further characterization revealed two different molecules, which were able to exert growth-hormone like effects on cartilage explants in vitro. Sequence analysis revealed a significant homology with pro-insulin, and hence they were termed insulin-like growth factors I and II (IGF-I and IGF-II) (Rinderknecht and Humbel, 1978 a, and b). Both IGF-I and IGF-II are synthesized as prohormones (9 kDa and 14 kDa respectively) and undergo posttranslational cleavage and glycosylation to achieve their 7 kDa active form. Although IGF-I and IGF-II elicit very similar biological responses there are significant differences in their pattern of expression in vivo. In mammals, IGF-I is preferentially expressed after birth and is produced almost exclusively in the liver. IGF-II is preferentially expressed in early embryonic and fetal development in a wide variety of somatic tissues. The adult expression of IGF-II takes place in the liver and in the epithelial cells lining the surface of the brain, i.e. the meninges and the choroid plexus.

Both IGF-I and IGF-II are present in the circulation and can be readily detected in plasma. As might be predicted from the pattern of synthesis, circulating IGF-I levels rise during juvenile life and then decline after puberty, while circulating IGF-II levels are highest in the fetal circulation (Sara et al., 1983). Circulating IGF:s are not floating free in the plasma but are associated with a set of specifically designed binding proteins. Over the last decade an increasing number of IGF-binding proteins (IGF-BPs) which exhibit tissue-and stage-specific expression, have been described. In vitro all IGF-BPs inhibit the biological activity of free IGF:s, suggesting that part of their function may be to restrict the availability of biologically active IGFs (Rechler, 1993).

Moreover, the gene for IGF-II is parentally imprinted (de Chiara et al., 1992). In fact this constitutes the basis for an ingenious regulatory mechanism since the type II IGF receptor, which is generally believed to act as an IGF scavenger, is oppositely imprinted and thereby contributes to a finely tuned control of IGF-II availability (Haig and Graham, 1991).The human IGF-II gene consists of nine

exons and the fetal expression is driven from three different promotors giving rise to a multitranscript family (Schofield and Tate, 1987). At birth there is a switch in that the fetal promotors are down-regulated and an adult promotor located 5´of the first exon is activated. This promotor has recently been shown to be active from the sixth week of gestation (Parkin, 1995). However, in human adult life IGF-II is almost exclusively expressed in the liver and the choroid plexus (Ward and Ellis, 1992).

*The transforming growth factor beta (TGFβ) family*

The TGFβ:s are homodimeric, disulphide-bonded proteins of approximately 25 kDa made up from two 12 kDa polypeptide chains. Five structurally related TGFβ genes exist namely TGFβ 1, 2, 3 in mammals, TGFβ-4 in chicken and TGFβ-5 in Xenopus. In mammals TGFβs 1-3 are widespread in their expression, TGFβ-1 being the most abundant. In several tissues the different TGFβ:s are co-expressed. TGFβ is secreted as a pro-form and is activated by proteolytic cleavage. The TGFβ 1-3 differ most significantly in the pro-region of the molecule.

The TGFβs act as modulators of other growth factors and in this context they can either enhance or abrogate the stimulating effect of other growth factors on quiescent cells in vitro. The action of TGFβ clearly depends on the physiological state of the responding cell and the presence of other growth factors. In some cases TGFβ induces cell proliferation whereas in other it is inhibited. In addition the biological function of the TGF:s is complex in the whole organism and highly dependent upon the exact context in which it acts.

The activation of TGFβ molecules appears to be complex and a pivotal role has been attributed to proteases as e.g. plasmin and cathepsin D in this process. Moreover, the type-II IGF receptor which in fact also is the mannose-6-phosphate receptor may play a key role, since the presence of three M6P molecules on the TGFβ precursor may help direct it to the intracellular sites where it can be enzymatically activated. TGFβ can stimulate as well as inhibit cell growth. One of the processes where it seems to be involved is morphogenesis and tissue remodelling. Furthermore, it is expressed in the embryo already at the 4 cell stage. However, these puzzling growth factors are thought to be even more complex in their action than hitherto demonstrated.

TGFβ 1-5 are members of an extended superfamily. These include the activin/inhibin family composed of homo- and heterodimers of two protein chains derived from three genes α, βA, and βB the family of bone morphogenetic proteins BMPs 2-7 and Mullerian Inhibitory Substance (MIS).

## Membrane receptors and their elicitation of cell proliferation and motility

The induction of DNA synthesis, cell locomotion and other biological effects of growth factors depend on the binding of the growth factors to specific high-affinity receptors on the plasma membrane of the target cells. The binding of the ligand to the extracellular domain of the receptor elicits a cascade of intracellular events which eventually lead to the onset of DNA replication. Growth factor receptors therefore not only confer cell-type specificity on the action of growth factors but also define the biochemical identity of the subsequent intracellular events. Stimulation of quiescent cells by specific growth factors, such as e.g. FGF leads to the rapid phosphorylation of the tyrosine residues of specific intracellular proteins. The molecular characterization of the cell surface receptors for a variety of growth factors has shown that the intracellular domain in all cases contain a stretch with tyrosine kinase activity, which is switched on by binding of the growth factor. The substrates for growth factor tyrosine kinases include the receptor itself and a number of key intracellular regulatory enzymes.

Identification and characterization of the receptors for a significant numbers of growth factors has revealed additional receptor species containing intrinsic ligand-activated tyrosine kinase domains. These include five species of FGF receptor (FGF-R 1-5), the insulin receptor and the type-1 IGF receptor. Nearly all tyrosine kinase receptors hitherto described consists of an extracellular ligand-binding domain, a hydrophobic trans-membrane domain, a region containing the tyrosine kinase, and a carboxy terminal extension extending into the cytoplasm of the cells.

Some tyrosine kinase receptors, including members of the FGF receptor family have split kinase domains in which the region encoding the enzymatic activity is interrupted by an insertion sequence. The design of the extracellular ligand-binding domain also varies from species to species. In the FGF-R family the extracellular ligand-binding domain is structurally related to the immunoglobulin gene superfamily. Finally, while most tyrosine kinase receptors have a single transmembrane domain, the insulin and IGF type-I receptor are heterodimers composed of two identical extracellular ligand-binding domains linked through disulphide bonds to a homodimeric transmembrane protein containing the tyrosine kinase domain.

It has been believed for some time that tyrosine phosphorylation must play a pivotal role mediating regulatory signals within the cell. Activation of the kinase activity presumably leads to tyrosine phosphorylation of certain substrates whose biological activity is readily altered. In particular IRS-1 (and presumably also IRS-2) act as interfaces between signalling proteins and the membrane receptors, leading to further downstream processes which ultimately result in the onset of DNA replication and mitosis (Sun et al., 1995). Four target proteins which will serve as physiological tyrosine kinase substrates have been proposed. The first of

these is the enzyme phospholipase Cγ (PLC-γ), which cleaves inositol-linked phospholipid (PIP2) to produce inositol derivatives (IP3) and diacylglycerol (DAG). The second is an enzyme phosphoinositol 3-kinase (PI-kinase) which phosphorylates the inositol phosphate sugar in the 3 position. In both these cases receptor-mediated phosphorylation of the enzyme results in a distinct increase in enzyme activity. The third proposed protein is a threonine/serine kinase, c-raf, which was first identified as a proto-oncogene, and the fourth is ras-GAP, a protein required for GTP:ase activation.

Very little is known about the molecular nature of the TGFβ superfamily of growth factors. However, cloning and characterization of the receptor for activin uncovered a novel class of receptor with serine/threonine kinase activity. phylogenetically conserved transmembrane Although nothing is currently known about the substrates for these receptor kinases it seems very likely, based on understanding of other growth factor receptors, that other members of the TGFβ superfamily will prove to act through a related set of receptors.

Different cell types respond differently to growth factor binding to membrane receptors. The range of biological responses includes proliferation (i.e. DNA synthesis and mitosis as well as protein accumulation and cellular enlargement), differentiation, locomotion, cell survival and functional modulation. Whereas the effects of growth factors on initiation of DNA-synthesis was studied in a vast number of systems and their precise roles often pinpointed, the effects on cell locomotion were not properly recognised until much later. The potential role of growth factors in in vivo processes as embryonic development, angiogenesis, tissue repair and tumour invasion and metastasis eventually brought about an interest for cell locomotion. Moreover, the notion that one growth factor could exert different biological effects at different concentrations began to emerge after a pioneering study by McAvoy and Chamberlain (1989), who by applying different concentrations of bFGF to chick lens cells could provoke as different responses as proliferation, motility and differentiation. Whereas a general locomotive effect of growth factors was discussed by Brunk et al. (1976) and Collins et al. (1979), it was subsequently found that malignant cells could be preferentially stimulated to motility by addition of a single growth factor (Westermark et al., 1982, Gregoriou et al., 1984, Engström 1986).

To date several motility promoting growth factors including transforming growth factor β (Morton et al., 1995), Insulin like growth factor I (Ziegler et al., 1996), Leukemia Inhibitory Factor (Granerus et al., 1994) and at least three members of the FGF family (aFGF, bFGF and K-FGF) (Mignatti et al., 1991, Pienta et al., 1991, Boyer et al., 1993, Chichoine et al., 1995). In the case of bFGF induced cell locomotion it was recently possible to link this particular effect to one receptor (Johnston et al., 1995), which may well assume that in the case of FGF-induced activity different receptors mediate different responses.

## Apoptosis

Apoptosis is a morphologically distinct form of programmed cell death, which is observed during embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, and normal tissue turnover (Wyllie, 1993). It plays a major role in development and homeostasis and the failure of cells to undergo apoptosis is believed to be an integral part of pathological conditions including cancer, autoimmune diseases and viral infections (Waux et al., 1992; Bursch et al., 1992). Other diseases characterized by cell loss, such as neurodegenerative disorders, AIDS, muscular dystrophy (Smith and Schofield, 1995) and osteoporosis, may be a result of increased rates of apoptosis (Thompson, 1995).

The capacity to undergo apoptosis seems to be a hallmark of all mammalian cells, but the activation of the immediate suicidal programme is regulated by many different extracellullar as well as intracellular signals.

During apoptosis the nucleus and the cytoplasm condense, and the cell often fragments into membrane-bound apoptotic bodies, and is rapidly phagocytosed by macrophages or other neighbouring cells (Wyllie et al., 1980, Arends et al., 1991). Endonucleases are activated to degrade chromosomal DNA and as a rule DNA is degraded into fragments the size of oligonucleosomes, but also larger fragments (50 to 300 kb) can be produced (Wyllie, 1980).

Apoptosis is by definition distinct from necrosis, a pathological form of cell death, when the cell dies as a result of hypoxia or toxic damage and the release of cytoplasmic material often triggers an inflammatory response. In the case of apoptosis the efficient elimination of the dead cells probably prevents any response from the immune system.

Within each organism the control of cell numbers is determined by a balance between cell proliferation and cell death. Cell proliferation is a highly regulated process with numerous control points during the cell cycle (Zetterberg, 1996). Activation of growth factor genes and proto-oncogenes are positive regulators, while tumour suppressor genes act as inhibitors and prevent cell cycle progression by inhibiting the activity of proto-oncogenes (Bishop, 1991).

The regulation of cell death is just as complex as the regulation of cell proliferation. A variety of extrinsic and intrinsic signals are involved in the decision of whether a cell will die or survive. Growth factor withdrawal, treatment with glucocorticoids, activation of certain oncogenes, chemotherapeutic drugs and gamma radiation are some of the signals that are capable of inducing a cellular suicide. The rational being a defence mechanism to remove unwanted and dangerous cells.

Apoptosis is also important during embryogenesis in complex organs where a subpopulation of cells are selectively killed. For example, from the many

neurones, produced in excess in the vertebrate central and peripheral nervous system, up to 50% or more of them die by apoptosis and self-reactive T-cells are eliminated within the thymus. Where tissue remodelling is prevalent, apoptosis is again an important factor in the generation of form, for example in the limb. In the adult, apoptosis can be seen in tissues undergoing reversible expansion, for example in hormone dependent cells of the breast and prostate after hormone removal (Sandford et al., 1984). Moreover apoptosis is the mechanism by which the immune system can kill infected cells and tumour cells.

Molecules essential for the induction of cell death, its regulation and the engulfment of dead cells have been best defined in the developing nematode *Caenorhabditis elegans*. This organism has been preferentially used because of its genetic homogeneity and small genome. In this nematode 131 out of 1031 somatic cells die in every organism. Mutation analysis has revealed the existence of 14 genes called *ced genes* (cell death defective) that are active during apoptotic cell death in the nematode. Three genes have been shown to affect the execution of the cells, *ced-3*, *ced-4* and *ced-9*.

*ced-3* and *ced-4* are required for cell death to occur. If either gene is inactivated by mutation, the cells that should normally die survive instead. The *ced-3* protein is closely related to the cysteine protease *interleukin-I beta converting enzyme, ICE*, a death effector molecule previously identified in vertebrates (Wang et al., 1994).

The *ced-9* gene which is a negative regulator of cell death, is required to protect cells that should survive (Hengartner et al., 1992), The gene seems to function as an inhibitor of the *ced-3* and *ced-4* activity. The *ced-9* gene bears a significant homology to a mammalian oncogene, *bcl-2*. Recent data suggests that *bcl-2* is only one member of a family of related genes (Lam et al., 1994). The mammalian protein, *Bcl-2*, acts like *ced-9*, to suppress apoptotic cell death in many types of mammalian cells.

*bcl-2* was originally cloned from the translocation breakpoint between chromosomes 14 and 18, found in follicular B-cell lymphomas. It has been shown that *bcl-2* expression rescues lymphoid cells otherwise determined to die from withdrawal of the cytokine *interleukin-3* (Wang et al., 1988).The cells did not proliferate, but the *Bcl-2* protein acted as a survival factor, not as a mitogen.

Expression of human *bcl-2* can inhibit apoptosis in *C. elegans*, and even partially substitute for the loss of *ced-9* function. Since two of the three genes that are known to be involved in the apoptotic death program in nematodes, *ced-3* and *ced-9*, are similar to mammalian apoptotic genes, it is likely that similar genes operate during apoptosis in all animals, and that the mechanism and the regulation of apoptotic cell death have been highly conserved during evolution.

An intracellular partner of *bcl-2* - *bax*, has been identified and further studies have indicated that *bax* regulates the antiapoptotic effects of *bcl-2*. The survival factor function of *Bcl-2* may depend on its ability to heterodimerize with the *Bax* protein, but increased levels of *bax* expression seems to counteract the survival effect of *Bcl-2* and promote and accelerate, rather than inhibit, apoptosis (Oltvai et al, 1993 and recently reviewed by Fraser and Evan, 1996).

Another suppressor gene, *p53* has been identified as a central activator of one of the pathways to mammalian apoptosis (Marshall, 1991). The gene product is necessary for cells to initiate apoptosis if the cells are exposed to ultraviolet light, to gamma rays or other genotoxic damage. The damaged cells react by raising the levels of *p53* protein which blocks cell proliferation. The cells either delay in $G_1$ phase or die by apoptosis. Humans who, due to mutation or inheritance, only have one functional copy of the *p53* gene are strongly predisposed to develop cancer.

The hereditary disease Li-Fraumeni syndrome is a rare disease, where the patients develop several independent tumours in a wide variety of tissues. The tumour cells in these patients have defects in both copies of the *p53* gene, while the non-tumour cells have defects only in one copy. One functional *p53* gene evidently protects the cells from being transformed.

It has been observed that tumours, deficient in *p53* have enhanced resistance to chemoterapeutic drugs and radiation, depending on the inability of the cells to undergo apoptosis. In mice lacking functional copies of the *p53* genes, thymocytes cannot undergo irradiation induced apoptosis. However, thymocytes from such mice undergo apoptosis in the expected fashion in response to gluco-corticoids or signals that act through the T-cell receptor. Moreover, in the developing mouse nervous system it has been shown that the loss of another tumour suppressor gene - *Rb* - activates both *p53* dependent and *p53* independent pathways towards apoptosis (Macleod et al., 1996). Taken together, these findings suggest that genes involved in one cell death pathway may not necessarily play an essential role in other pathways. Most probably the apoptotic cell death is a more common phenomenon than hitherto believed. In most tissues cell survival appears to depend on the constant supply of autocrine and paracrine survival factors, provided by the neighbouring cells and the extracellular matrix. Most cells will undergo apoptosis if cultured in the absence of exogenous survival factors.

In conclusion, it seems reasonable to assume that cells are actively kept alive by a complex web of survival factors acting in a paracrine fashion. Most cells are programmed to commit suicide if survival signals are not received from the environment, either constantly or at regular intervals.

# Materials and methods

*Tumour material*

The testicular tumour samples used for the studies in paper I and IV, were obtained from patients after orchidectomy. We used normal testis (control), testicular tumour tissues, e.g. seminomas, embryonal carcinomas, teratomas, as well as adjacent tumour-free testicular tissues.

*Growth factors*

The growth factors human acidic fibroblast growth factor (aFGF), human basic fibroblast growth factor (bFGF) and the human transforming growth factors β 1-3 (TGF β 1-3) were purchased from British Biotechnology, Oxford, UK. Recombinant human insulin-like growth factor I (IGF 1) was purchased from Amersham, UK and from Kabi, Pharmacia, Sweden. Human insulin-like growth factor II (IGF-II) was a gift from Kabi, Pharmacia. Protamine sulphate was purchased from Sigma, Poole, UK. Transferrin was acquired from Boehringer Mannheim, Sweden. Tritiated thymidine, (56mCi/mM) was obtained from Amersham, UK.

*The Tera-2 cell line*

The human embryonal carcinoma cell line Tera-2 was originally established in 1971 by Dr. Jörgen Fögh (Fögh and Trempe, 1975) from a pulmonary metastasis of a primary testicular teratocarcinoma. In 1984, three clones (clone 5, 12 and clone 13) were isolated and characterized from the human Tera-2 cell line (Thompson et al., 1984). Briefly it was found that the karyotypes of these clones were all abnormal; they possessed an aneuploid karyotype with a near triploid number of chromosomes. The undifferentiated, progressively growing cells were characterized by lack of expression of P2-microglobulin and HLA-A, B, C determinants. The expression of these determinants was studied with the monoclonal antibodies W6/32, PA 2.6 and anti-β2, The undifferentiated cells rarely reacted with these antibodies. Conversely the human EC-cell specific antibody GCTM-2 was used to confirm the undifferentiated status. Most of the cells expressed Thy-1 antigen and a human glycoprotein, present on human brain and white blood cells. The monoclonal antibodies used were F15.42.1 and F10.44.2 respectively. Approximately half of the cells expressed sugar determinants detected by the anti-SSEA-1 antibody. The cells lacked reactivity with reagents that mark cells in the nervous system.

These cells were also shown to be tumourigenic. Andrews et al. 1984 injected Tera-2 cells into nude mice. The cells gave rise to teratocarcinomas 2-3 months after inocculation. Primitive epithelium and neurons could be detected in the

tumour as well as EC. This and other evidence suggests that Tera-2 is a pluripotentional stem cell line, which at least in part reflect an early human embryonic stem cell. Similar observations, with different clones of Tera-2 have been made by Andrews (1984).

In response to treatment with retinoic acid, applied to cells in monolayer culture, the small monomorphic stem cells of Tera-2, could be induced to differentiate into a mixture of cell. types, expressing characteristic markers. One of the most easily immunohistochemically identifiable were neuron-like cells that expressed tetanus toxin receptors and neurofilaments. Those cells reacted with tetanus toxin, with the anti-neurofilament antibodies BF10 and RT97 and also with the anti-ganglioside, GQ 1c antibody F12 A2B5, and anti-Thy-1.

Most of the differentiated cells expressed β2-microglobulin and HLA-A, B, C polypeptides. About half of the population still expressed SSEA-1 sugar determinants, detected by the anti-SSEA-1 antibody. On differentiation there was a decrease in the proportion of cells that expressed Thy-l and the glycoprotein recognized by the antibody F10.44.2. The expression of HLA-A, B, C antigens on the cell surface and

of β2-microglobulin was studied with the monoclonal antibodies W6/32, PA 2.6 and anti β2. The synthesis of fibronectin was also examined, since this protein often is expressed as human teratoma cells differentiate (Andrews, 1982). The anti-fibronectin antibody 2.3 F9 was used to study the difference in expression of this protein between differentiated and undifferentiated Tera-2 cells. It was found that all the differentiated cultures secreted more fibronectin in comparison with the undifferentiated cell cultures.

The majority of the cells in a population treated with retinoic acid, turned from a rapidly dividing state, with nuclear DNA amounts up to 8C, into a population where the cells were arrested in the $G_1$ phase of the cell cycle, and the DNA amounts not more than 3C within 10 days.

## Cell culture

*Stock culture*

The Tera-2 clone 13 used in these studies, was a gift from Prof. C.F. Graham. All experiments were performed on cells between the 20$^{th}$ and 30$^{th}$ passage after cloning.

The cells were grown in alpha-modified Eagle's medium, lacking nucleosides and deoxynucleosides (alpha-MEM, Gibco, Europe), containing 10% (v/v) heat-inactivated fetal calf serum, 50 units of penicillin and 50 microgram of streptomycin sulphate/ml medium In order to make the cells attach firmly to

tissue culture surfaces, the culture bottles were precoated for 2 hours at 5°C with gelatine 0.l-2% solution (w/v) (swine skin, Typel gelatine, SIGMA), in calcium/magnesium free phosphate-buffered saline (PBS). The cells were grown under humidified 5% (v/v) $C0_2$/95% air at 37°C. Stock cultures were maintained in 25 $cm^2$ plastic tissue culture bottles (NUNC, GIBCO) and subcultured every 5 or 7 days at a routine split ratio of 1 in 4. The cells were removed from the bottle before transfer, by treatment with 0.125% trypsin in $Ca^{2+}$/$Mg^{2+}$-free PBS supplemented with 50 mM EDTA (Dulbecco and Vogt, 1954). At each passage samples were frozen in 10% dimethylsulpoxide (DMSO) 90% fetal calf serum (v/v) and stored in liquid nitrogen at -70°C.

Serum-free medium was composed of a 50/50 mixture of alpha modified Eagles medium, lacking nucleosides and deoxyribonucleosides, and Ham's F 12 medium (GIBCO, UK), supplemented with 10 μg/ml human transferrin, preloaded with iron in accordance with the rnanufacturers instructions.

*Induction of differentiation*

Undifferentiated cells were routinely plated at $1x10^5$ cells per 90 mm dish for growth assays. Induction of differentiation was achieved by plating cells in the presence of $2x\ 10^{-5}$ M all trans retinoic acid (Eastman Kodak) diluted from a 0.1 M stock in dimetylsulphoxide. Cells were harvested by rinsing with calcium/magnesium-free phosphate buffered saline and taken from the dishes with solution of 0.125% (v/v) trypsin in PBS containing 0.5 mM EDTA. The cells were spun in Eppendorf tubes to give approximately $1x10^6$ cells per tube. The cells were then incubated in 20 μl of monoclonal antibody W6/32 culture supernatant that had been diluted previously to 1/10 of its original volume with calcium/magnesium-free PBS containing sodium azide and 2% (v/v) fetal calf serum. After incubation for 45 minutes at $4^C$, 0.5 ml of the above azide/serum/PBS solution was added and the samples spun. The cell pellet was resuspended in 20 μl of a 1/30 dilution of rabbit antimouse immunoglobulin G-fluorescein isothiocyanate for 45 minutes and viewed under a fluorescence microscope. At least 200 cells were counted for each sample.

The human EC-cell specific antibody GCTM-2 (Pera et al., 1988) was used as described at a final dilution of 1:50 on cells grown in parallel to experimental cultures on coverslips gelatinised by overnight treatment with 0.5% (v/v) gelatine (Sigma).

*Growth assays*

To assess cell numbers in culture, the cells were grown for two days in alpha/Ham supplemented with 10% fetal calf serum, to allow the cells to adapt to the basal medium used in the serum-free culture experiment. Cells were harvested by rinsing twice in phosphate buffered saline (PBS) and in serum-free alpha MEM,

and then briefly exposed to trypsin. Cells were taken up in 10% fetal calf serum, collected by centrifugation and counted in a haemocytometer. Cells for growth curves were then plated out at a density of 1.4-3 x $10^5$ cells in each 6 cm diameter Primaria dish (Beckton-Dickinson, UK) and allowed to attach for 24 hours in 10% fetal calf serum alpha/Ham. Cultures were then washed and the medium with fetal calf serum was then replaced with serum-free medium. The following day the cells were counted, and this was taken as the starting point of the experiment. Counting of cells exposed to experimental conditions was carried out after exposure of washed monolayer to trypsin/EDTA for 20 minutes at room temperature to ensure complete harvesting. The range of cell counts in the duplicate and triplicate dishes rarely exceeded 10% of the mean cell count.

In some experiments the cells were seeded onto gelatine coated glass coverslips in 35 mm plastic Petri dishes at a concentration of $10^5$ cells per dish. Cells were allowed to attach and were thereafter maintained in 10% fetal calf serum alpha-Ham for 72 hours before each experiment. The cells were then washed and the medium replaced with serum-free medium.

*Clonal analysis*

The cells were plated at different densities ranging from 10-100 cells per $cm^2$ into equilibrated medium in 35 mm wells of six-cluster dishes. After 5 days, each culture was fixed in 3:1 methanol/acetic acid for at least 24 hours, hydrated in Analar water, and finally stained with crystal violet. Each dish was examined under a Leitz inverted microscope, equipped with an eyepiece graticule inscribed with 10 concentric circles. The distance between two circles was taken as one relative unit. Image analysis using a C-Scan (Cambridge) analyser allowed for quantitation of colony size in parallel and gave identical results to the manual method.

Each dish was scanned in the microscope and the location of each colony was marked. The diameter of each colony was determined by use of the graticule, whereafter the number of cells in each colony was counted. In several instances the colonies were photographed under a Leitz inverted microscope with an attached camera system.

*Determination of the proportion S-phase cells*

Tera-2 cells growing on coverslips were exposed to media with the various growth factors specified for 24 or 48 hours. During the last hour prior to fixation, the cultures were exposed to 10 μCi tritiated thymidine (Amersham 56 mCi/mmol) for 30 minutes and thereafter washed in PBS and subsequently fixed in 95% ethanol. After fixation the slides were washed in a 10% (w/v) solution of trichloroacetic acid for 30 min at 4°C, followed by three successive washes in distilled water. The coverslips were then drained and coated with Ilford K2

emulsion diluted 1:1 (v/v) with 2% glycerol in distilled water, dried and exposed at 4°C for 7 days. After this time reduced silver grains were developed in Kodak D I9 developer for 5 min, washed in distilled water and fixed with Kodafix (Kodak) diluted 3:1 (v/v) with distilled water according to the manufacturer's instructions. After extensive washing in tap water the coverslips were air dried and stained in 1% /w/v) crystal violet. The proportion labelled cells was determined by light microscopy.

## FGF receptor assays

*Binding - Displacement assays*

Radioiodinated FGF was prepared according to Neufeld and Gospodarowicz (1986), and purified by heparin Sepharose affinity chromatography. Specific activities of 4-8 x $10^4$ cpm/ng were obtained in this way.

Tera-2 cells were plated out on gelatinised 24-cluster plates at $2x10^5$ cells per well in alpha MEM/10% fetal calf serum and allowed to attach and grow over night. Plates were transferred to ice, medium aspirated and cells washed 5 x with 500 µl phosphate buffered saline. Subsequently 200 µl of binding buffer (alpha MEM +25 mM HEPES + 0.2% gelatine) were added to each well together with increasing concentrations of labelled FGF (0.01-10 ng/ml). Non-specific binding was estimated by including 2 µg/ml unlabelled FGF with duplicate samples to which had been added 0.5 ng of iodinated bFGF/ml and binding to dishes by adding 5 ng of iodinated bFGF/ml to dishes without cells in 200 µl of binding buffer. Plates were incubated at 4°C for 4 hours, medium aspirated and cells washed with 0.5 ml PBS/0.1% BSA three times. Membranes were then solubilised with 400 µl of Triton X-100 + 0.1% BSA and counted in an LKB rack gamma counter. Greater than 95% of counts bound to the cells on incubation with 0.5 ng/ml of iodinated FGF. The binding could be displaced by 2 µg/ml of unlabelled competitor in repeat data sets. This was taken as a measure of non-specific binding and subtracted from the binding data. This data was subsequently converted to the Scatchard (Scatchard, 1949) form and analysed using the programme LIGAND (courtesy of Dr. AR. Rees, Oxford). All figures were corrected for non-specific binding as described above.

*FGF receptor crosslinking*

Receptor cross-linking was carried out essentially according to Neufeld and Gospodarowicz (1985). Tera-2 cells were plated out onto gelatinised 600 mm dishes and allowed to attach over night. Binding was carried out at 4°C in 1.2 ml of the binding buffer of Neufeld and Gospodarowicz (1986). 100 ng/ml of iodinated FGF was added to each dish. Competitor was added at 2 µg/ml to one set of dishes and increasing concentrations of protamine sulphate (1.5 and 10 µg/ml) to the remaining dishes. Dishes were incubated for 2,5 hours on a rotating

platform at 2 cycles per second, washed in 10 ml of PBS and crosslinked at room temperature with 20 mM disuccinimidyl suberate (Aldrich) in 2 ml of PBS. Harvesting of membranes was carried out as described in Neufeld and Gospodarowicz (1985), and analysed on 5% Laemmli SDS-polyacrylamide denaturing gels using the Amersham (Amersham, UK) Rainbow markers for calibration.

## Gene expression analysis

*Isolation of mRNA*

Cells were washed with 5 ml of pre-warmed PBS and lysed in 2 ml per dish of 4 M guanidinium thiocyanate (Fluka) 25 mM trisodiumcitrate (pH 7.0), 0.1 M β-mercaptoethanol, 0.5% Sarkocyl (BDH) (GuSCN-buffer).

Tissue samples from testicular tumours were homogenized in GuSCN-buffer with a high speed Ultra Turrax homogenizer. The lysates were layered onto a 2.2 ml cushion of 5.7 ml CsCl, 0.1 M EDTA (pH 8.0) in a Beckman SW 40 Ti rotor for 20 hours at 18°C. The resulting pellet was washed, taken up in 100 ml of filtered diethylpyrocarbonate-treated water and ethanol precipitated twice. Poly A+ RNA was extracted by the use of oligo (dT) cellulose (Pharmacia type 7) as described in Hyldahl et al. (1990).

*Northern blotting*

Gel electrophoresis of Poly A+ RNA was performed according to Lehrach (1977).

The RNA was transferred to a Biodyne A nylon membrane (PALL, UK) by blotting overnight in 20X SSC (IX SSC = aqueous 0.15 M NaCl, 0,02 M sodium citrate, pH 7,0). The blots were washed, air dried, baked for 4 hours at 80°C, prehybridized overnight with 250 μg/ml of sonicated and denatured salmon sperm DNA in 5X SSC, 50 mM phosphate buffer at Ph 6.8, 5x Denhardt's solution, 0.1% (w/v) sodium dodecyl sulphate (SDS) and 50% (v/v) deionized formamide. They were hybridized with the labelled probe for at least 48 hours at 42°C.

*cDNA probes*

The probes used in the studies were :

The K-FGF/hst probe was a 285 bp SacI/Hind III fragment from pORF1 provided by Prof Takashi Sugimura. The glyceraldehyde 3-phosphate dehydrogenase probe was murine cDNA, a gift of Dr. Peter Curtis, Wistar Institute, Philadelphia. The cDNA probe for human c-myc was a gift of Dr. Natalie Teich, ICRF, London.

## Assessment of cell death

*Determination of nuclear morphology*

The assessment of intact versus apoptotic cells were performed by acridine orange staining. The glass coverslips with the attached cells were rinsed in prewarmed PBS, and instantly stained with 1% acridine orange. They were examined in a Leitz fluorescence microscope at 435 nm and photographed.

*Determination of chromatin degradation*

Two 75 $cm^2$ tissue culture flasks of Tera-2 cells per experimental group were incubated with 0.25% trypsin at room temperature until cells just detached. The trypsin activity was stopped by adding I% (v/v) final concentration soybean trypsin inhibitor. The cell suspension was collected by low speed centrifugation, and high molecular weight DNA was isolated according to Pesce et al. (1993). The DNA extract was normalised to cell number and μg aliquots were electrophoresed on an 0,8-2% agarose gel in Tris/acetate/EDTA. Gels were stained with ethidium bromide and visualized by ultra-violet illumination.

## Insulin-like growth factor determination in tumour samples

The testicular tissue samples were minced in 1M acetic acid in ethanol (1:4 weight of tissue volume), sonicated for 10 seconds, then spun at 2000 x g for ten minutes. The supernatant was removed and frozen at -29°C until further use. The acid ethanol extracts of testicular tissues were equilibrated in 1M acetic acid to separate immunoreactive IGF from their binding proteins, and chromatographed on a Sephacryl S-100 HR column (Pharmacia) at a flow rate of 4-5 ml/hour. Fractions equivalent to a kd of 0.5-0.87 were analysed by IGF-I or IGF-II radioimmunoassay. IGF-I was measured using truncated IGF-I as a ligand and polyclonal antibodies raised against human IGF-I (gift of Prof. P. Gluckman, Auckland, New Zealand). IGF-II was measured in an assay using recombinant IGF-II (gift of Kabi, Pharmacia, Stockholm), and hen yolk antibodies, as described in Schofield et al. (1994).

# Results

## Basic characterization of Tera-2 clone 13

Undifferentiated Tera-2 cells in monolayer culture multiply exponentially with an approximate population doubling time of 24 hours (Schofield et al., 1987). In contrast, if cells are plated in the presence of 2 x105 M retinoic acid, overall cell numbers cease to increase by 12 to 18 days and the majority of cells become arrested in G1, as estimated by cytophotometry (Thompson et al., 1984). In the experiments described here, less than 6% of the undifferentiated cells expressed W6/32 reactivity (n>200), and the general lack of expression of HLA- A, B, C common determinants is consistent with their undifferentiated phenotype. After addition of retinoic acid, the cell cycle rapidly lengthens (Engström, W., unpublished observation). Paper 1 gives an estimate of the rate of aquisition of HLA- A, B, C common determinants during a fourteen day exposure to retinoic acid. After 12 days approximately 40% of the cells expressed antigens that reacted with the W6/32 antibody, and after 13 days 80% of the cells were positive. These figures were confirmed by staining populations of undifferentiated and differentiated (15 days) cells, using the antibody GCTM-2, which reacts only with a heparan sulphate proteoglycan on the surface of teratocarcinoma stem cells (Pera et al., 1988). The undifferentiated component of the 15 days RA-treated population was found to be less than 10%.

## Expression of genes potentially involved in growth control

*K-FGF*

Tera-2 cells induced to differentiate

Poly-A+ RNA was prepared from cultures exposed to retinoic acid for 60 minutes, 5, 12 and 15 days together with samples of undifferentiated cells. The K-FGF transcript had almost disappeared after 60 minutes exposure to retinoic acid, indicating a very rapid down-regulation of this gene. This findings confirm a previous preliminary report (Schofield, 1989), and is also consistent with a study reporting that another human embryonal carcinoma cell line, NT2/D1, downregulates K-FGF expression after 5 days exposure to retinoic acid (Tiesman et al., 1989). However, if the cells were exposed to retinoic acid for an extended period of time (i.e. 15 days), the 3 kb K-FGF transcript reappears. This finding has not been previously reported and suggests that K-FGF gene expression may represent a multifaceted function in the control of growth and differentiation. By using two separate biochemical criteria for differentiation, it is clear, that the reappearance of transcript after 15 days of differentiation, is not the result of a small RA unresponsive undifferentiated population taking over the cultures. Addition of retinoic acid in different patterns, either once at the initiation of differentiation or on alter-

nate days through the experiment, did not affect the results (data not shown). It has been shown that K-FGF expression in the embryo is stage-specific and our observations on an embryonal carcinoma cell line in vitro, may well indirectly reveal crucial embryonic developmental pathways.

Testicular tumours

To put the expression of K-FGF in Tera-2 cells into perspective and to compare it with the phenotype of primary germ cell tumours, we purified polyA+ RNA from 10 surgical specimens of human testicular tumours (9 seminomas and 1 embryonal carcinoma), as well as from normal testis. The expected 3 kb transcript was observed in two seminomas and one embryonal carcinoma. No K-FGF transcript could be detected in 7 seminomas or in normal testicular tissue. This data confirm a preliminary report by Yoshida et al. (1988), who failed to detect transcripts from this gene in any other tissue but in five human germ cell tumours. Taken together these data suggest that K-FGF may be important in embryonal stem cell growth and may contribute to the development in germ cell tumours.

*c-myc*

Tera-2 cells

We have previously reported, that high levels of expression of c-myc predisposes cells to programmed cell death in the absence of permissive factors such as serum, or perhaps other growth factors (Granerus et al., 1994; Harrington et al., 1994). It has also been reported that Tera-2 show massive levels of c-myc expression (Schofield et al., 1994), both in its proliferative and differentiated states, and it seemed important to know if levels of c-myc expression remained elevated under conditions of serum deprivation where massive apoptosis occurs.

Poly A+ RNA from Tera-2 cells was subjected to northern blotting and hybridized with a cDNA probe to human myc. Figure 5, paper 3, shows the expression of the c-myc proto-oncogene in Tera-2 cells after 24 hours exposure to 10% serum, serum-free medium, or serum-free medium containing 10 ng/ml of IGF-II. It can be seen, that the intensity of the major transcript is unchanged, irrespective of the composition of the medium, indicating that in these cells, myc is unresponsive to exogenous growth factor provision. We also found that the transcriptional activity was neither enhanced nor decreased by addition of IGF-I or TGFβ-1. In all cases, the filters were stripped off bound probes and rehybridized with a cDNA for GAPDH, a house-keeping gene, which is believed to be equally expressed in all cell types. This is a standard procedure to ensure equal loading of RNA into the slots on a gel used for expression analysis.

## The effect of growth factors on Tera-2 cells

*Effects on DNA-synthesis*

In order to compare the proportion of Tera-2 cell traversing the S-phase under different conditions, cell cultures were exposed to serum-free medium with or without supplementation of 10 ng bFGF, aFGF, TGFβ-1, TGFβ-2 or TGFβ-3 per ml. The proportion of cells, undergoing DNA replication at a given moment, was assayed by pulse-labelling with tritiated thymidine between the 23rd and 24th or the 47th and 48th hour after medium change and subsequent autoradiography. The results clearly indicates, that the percentage of 3H-thymidine labelled cells was virtually unchanged when Tera-2 cells were deprived of serum. The percentage of labelled cells in 10% serum was after 23-24 hours 35.1, after 47-48 hours 35.7. In serum-free (SFM) medium the percentage was 36.6 and 31.5 respectively.

*FGF:s*

The addition of heparin-binding growth factors to serum-free medium did not significantly alter the proportions of S phase cells. When 10 ng aFGF/ml was added to the medium, the percentage of labelled cells was 32.7 after 23-24 hours and 31.0 after 47-48 hours. The corresponding figures when 10 ng bFGF/ml was added, were 35.0 and 34.1%.

*IGF-I*

When the serum-free medium was supplemented with 10 ng/ml IGF-I the proportion of S -phase cells was not significantly altered. The labelling figures we got after 23-24 hours were 34.2%, and after 47-48 hours 30.3%.

*TGF:s*

None of the TGF:s did significantly alter the proportion of Tera-2 cells undergoing DNA-replication. Serum-free medium plus 10 ng/ml TGFβ-1 gave 33.8% labelled cells after 23-24 hours, 34.8% after 47-48 hours. Serum-free medium plus 10 ng/ml TGFβ-2, 33.8% and 37.9% respectively and finally serum-free medium plus 10 ng/ml TGFβ-3 gave after 23-24 hours 35.1% and after 47-48 hours 30.6% labelled cells.

## Effects on cell multiplication

*FGF:s*

Daily addition of recombinant basic FGF to Tera-2 cells, plated out in serum-free medium over a four day period, showed that proliferation of the cells could be supported by 20 ng/ml bFGF as a sole macromolecular additive, resulting in a 1.3

fold doubling in population over the time course of the experiment, as compared with controls which showed essentially no growth. The proliferative response increases with increasing concentrations of bFGF and reaches a maximum at 20 ng/ml. No further stimulatory effect could be achieved by adding higher doses of the growth factor. The effects of bFGF are inhibitable by low concentrations (10 μg/ml) of protamine sulphate (data not shown) as previously reported by Gospodarowicz et al. (1984), and inclusion of this agent in growth experiments, substantially reduced the biological effects of the bFGF, suggesting that the molecule is acting through similar receptors to those previously characterized.

Whereas Tera-2 cells growing in 10% serum undergo exponential growth, cell cultures in a serum-free medium are maintained at a steady-state level. When we examined the effects of heparin-binding growth factors on Tera-2 cells over a 24 hour period, it was found that 10 ng of acidic or basic FGF per ml serum-free medium exerted an observable effect on cell numbers. The effects of FGF:s were examined over a five day period under different conditions, and it was found that 10 ng/ml of aFGF or bFGF, added to serum-free medium, supported multiplication of Tera-2 cells.

*IGF:I*

It was found that lower concentrations of IGF-I exerted only minor effects on Tera-2 cells in serum-free medium over a 24 hours assay period, while the addition of 1-100 ng IGF-I/ml resulted in cell numbers twice as high as in serum-free control cultures. Over a 5 day period we found that 10 ng IGF/ml to serum-free medium supports the multiplication of Tera-2 cells. Similar results were obtained with higher concentrations of IGF-I (50-200 ng/ml, data not shown), but the multiplication stimulating effect never reached that of 10% serum.

*TGF:s*

No concentration of TGFβ-1 seemed to exert any stimulatory or inhibitory effect on Tera-2 cell number over a 24 hour period. Nor were any effects observed on cell numbers over a 5 day period when 10 ng/ml TGFβ-1 was added to the serum-free cultures. No other concentration of TGFβ-1 (0,01-100 ng/ml) was found to enhance or repress Tera-2 cell proliferation in serum-free medium.

Neither 10 ng/ml of TGFβ-2 or TGFβ-3 exerted any effect on Tera-2 cell numbers over a 24 hour period. Moreover, even when we added the three TGFβs in concentrations ranging from 0.1-100 ng/ml, alone or in combination, did we achieve any effects on Tera-2 cell numbers (data not shown). Nor was any effect on cell proliferation observed over a 5 day period when higher or lower concentrations (0.01-100 ng/ml) of the three transforming growth factors, alone or in combination, were added.

## Effects on cell survival

*FGF:s*

The effect of growth factor supplementation to a serum-free medium on survival in Tera-2 cells was measured by staining Tera-2 cells with acridine orange and judging the proportion of intact versus fragmented nuclei with a fluorescent microscope. It was found that serum withdrawal reduced the number of intact nuclei from 90 to 35% over the first 24 hours. Between the 2nd and 5th day the proportion of intact nuclei stabilized at approximately 40%. Addition of 10 ng/ml of either aFGF or bFGF resulted in a substantially higher proportion of intact nuclei over the entire 5 day period.

*IGF:s*

The appearance of nuclear fragmentation, characteristic of apoptotic changes, was sought for over increasingly long periods of serum deprivation. After 12 hours, approximate one fifth of the cells had undergone apoptotic changes as judged by nuclear morphology, this proportion increased with time. In contrast, cultures maintained in 10% fetal calf serum after equivalent washing contained less than 10% apoptotic cells. The population could be protected from apoptosis by the addition of 1-100 ng/ml IGF-II. The proportion of apoptotic cells fell from 70% to 40% when assayed over a 24 hour period. This effect was saturated by 10 ng/ml of IGF-II, indicating that IGF-II was not the only factor required to completely prevent cell death. The number of dying cells increases slightly with time and this effect could be ameliorated by the addition of 10 ng/ml IGF-I or IGF-II throughout the period. To confirm that the nuclei seen were apoptotic, high molecular weight DNA was prepared from cultures starved of serum or supplemented with 10 ng/ml of IGF:I or IGF-II. Whereas DNA from cells growing in serum appeared mainly as a high molecular weight band, cells exposed to serum-free medium showed a characteristic nucleosomal ladder. This ladder was less pronounced in cells exposed to serum-free medium supplemented with IGF-I or IGF-II. This results confirm the conclusion drawn from the acridine orange staining of cells and show that serum starvation generates a form of cell death characteristic of apoptosis.

*TGF:s*

Supplementation with 10 ng/ml of TGFβ-1, 2 or 3 did not in any way alter the numbers of intact nuclei in serum-free cultures. Nor did we observe any effects of any other TGFβ concentration (data not shown). When TGFβ-1, 2 or 3 was added together with 10 ng/ml of IGF-I, aFGF or bFGF there was no enhancing or suppressing effect on cell numbers or the proportions cells with intact nuclei. Neither of the TGFβ:s alter the growth characteristics of Tera-2 cells growing in 10% serum (data not shown).

## Effects on cell motility

*bFGF*

To study the relationship between cell multiplication and cell locomotion we seeded Tera-2 cells at clonal density and grew them with the addition of 10 ng/ml of bFGF. In this set of experiments the number of cells as well as the diameter was scored for each colony. In order to exclude the possibility that any observed effects on cell migration were a consequence of an increased rate of cell proliferation, the diameters of colonies that consisted of equal numbers of cells were examined in the presence and absence of bFGF.

It was shown that bFGF stimulates clonal cell proliferation as determined by cells/colony in a concentration-dependant manner. The maximum increase in cell numbers was observed at 10 ng/ml. Increasing the concentration over and above this slightly decreased cell numbers. Irrespective of colony size, as defined by numbers of cells per colony, only high doses of bFGF (100 ng/ml) significantly increased the colony diameter. In contrast, lower concentrations of bFGF (1-10 ng/ml) did not significantly increase the colony diameter (fig. 2 A-C). The stimulatory effect on cell locomotion was ascribable to bFGF since it could be completely abrogated by simultaneous addition of protamine sulphate (data not shown).

None of the IGF:s or TGF:s showed any significant stimulatory effect on Tera-2 cell motility (data not shown).

## Receptor phenotype

*FGF receptors*

It is important in this type of analysis to establish that the observed requirements are complemented by expression of the appropriate growth factor receptors. Whereas the IGF-receptors have been extensively characterized in Tera-2 cells (Engström et al., 1985, Biddle et al., 1988) no data were hitherto available on the existence of FGF-receptors in this cell line. Scatchard analysis of FGF binding data yields a total number of 22.500 and 200.000 sites per cell made up of two populations of sites with high and low affinity dissociation constants of 16.7 pM and 51 nM, the former accounting for approximately 22.500 sites per cell and the latter 200.000. Cross-linking of radioiodinated ligand to monolayers indicated a single species of receptor of Mr 150 kD to which ligand binding could be competed with an excess of unlabelled ligand or increasing concentrations of protamine sulphate. It is particularly important to note that the degree of protamine sulphate induced inhibition of binding of FGF to its receptor mirrors the dose-responsive relationship for inhibition of FGF stimulated growth thus providing good cicumstantial evidence that the growth effects are mediated through the 150 kDa receptor.

# Conclusions

The close connection between normal embryonic development and the development of tumours with the capacity to undergo multipotent differentiation, resulted in the early use of teratocarcinomas as a tool to study processes in the early mammalian embryo. Moreover the clinical importance of germ cell tumours in man, made it highly desirable to develop a human multipotent embryonal carcinoma cell system that could be used for in vitro studies. In the mouse the isolation of the embryonic stem cell and the development of the transgenic technology has made the use of murine embryonal carcinoma cells cultures for studies of embryogenesis superfluous. In the human, however, there is still a great need for a system that in the best possible way can be used to mimic vital embryonic processes. The human embryonal carcinoma derived Tera 2 cell is a multipotent cell which can be induced to undergo differentiation by exposure to retinoic acid or by xenografting in vivo. In the absence of a human embryonic stem cell (ES-cell), the Tera-2 cell line remains the best characterized and the best alternative available.

Growth factors elicit a wide variety of responses in Tera-2 EC cells in vitro. Three groups of growth factors, the Insulin like growth factors, Transforming growth factors β and the Fibroblast growth factors, have been of particular interest, not least because they are intrinsically expressed in Tera-2 as well as in primary tumours (Granerus et al., 1991, paper 1) and therefore have been proposed to be part of autocrine loops (Schofield et al., 1994).

Neither growth factor altered the proportion of cells in S-phase in comparison with a serum-free environment. Nor did growth factor addition alter the rate of DNA-replication, indicating that Tera-2 is a typical tumour cell line, incapable of leaving the cell cycle in the undifferentiated state. Both fibroblast growth factors (aFGF and bFGF) and insulin growth factors (IGF I and IGF II) promoted increases in cell numbers. However it could be firmly concluded that this was a sole effect on cell survival. Growth factor addition significantly decreased the number of cells undergoing apoptosis.

The fibroblast growth factors (aFGF and bFGF) exerted an additional effect on cell motility. Whereas physiological concentrations of bFGF enhanced cell survival and hence increases in gross cell numbers supraphysiological concentrations exerted a preferential effect on cell motility. Thus it was concluded that one growth factor is capable of inducing different biological responses at different concentrations. It is tempting to speculate whether, in the case of bFGF at least, the biological specificity is executed by the use of different receptor species with different modes of action. Another possibility is that the FGF availability might be modulated by some binding proteins. However, this possibility remains to be substantiated by experimental evidence.

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