Regulatory factors of embryonic stem cells

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Summary

The analysis of factors which regulate cell proliferation and differentiation in early mammalian development has been facilitated by the existence of cell lines derived from pluripotential stem cells of the early embryo; embryonal carcinoma (EC) cells and embryonic stem (ES) cells. EC cells have proved to be a useful source of embryonic growth factors. A potent mitogen, ECDGF has been isolated from EC cell conditioned medium. ECDGF appears to be a novel member of the heparin binding growth factor family. A remarkable feature of heparin binding growth factors is their ability to induce mesodermal differentiation in *Xenopus laevis* animal pole explants. ES cell differentiation *in vitro* is controlled by exogenous factors, in particular a novel differentiation inhibitory factor DIA. Controlled bipotential differentiation of ES cells can be achieved by exposing ES cells to different combinations of regulatory signals.

Introduction

It is becoming apparent from the study of a number of stem cell systems that soluble regulatory hormones and their associated cellular response mechanisms play a key role in coordinating and controlling both cell proliferation and developmental switching. The early phases of mammalian development are no exception. In this paper we discuss the expression of, and the response to, growth and differentiation factors by stem cells derived from the early mammalian embryo.

The direct analysis of growth and differentiation factor action in early mammalian development is complicated by two problems; at the time critical morphogenetic events occur *in vivo* the embryo is composed of a few thousand cells only and is sequestered in the relatively inaccessible environment of the maternal uterus. This means that direct biochemical analysis, by conventional techniques, of regulatory factor action and expression is very difficult. Furthermore, the embryo is composed of multiple cell types in close anatomical association. This renders direct experimental dissection of embryonic intercellular signalling processes complex. In order to begin to understand the identity of the signalling pathways that operate in the early phases of mammalian development *in vivo*, attention has focussed upon approaches which permit controlled biochemical analysis of embryonic polypeptide regulatory factors under experimentally favourable conditions. In this paper we shall consider two avenues that have been developed with this objective in mind: the use of multipotential cell lines derived directly, or indirectly, from pluripotential stem cells of the early embryo and the exploitation of species with experimentally accessible embryonic stages, in particular, amphibians. The expectation is that the information

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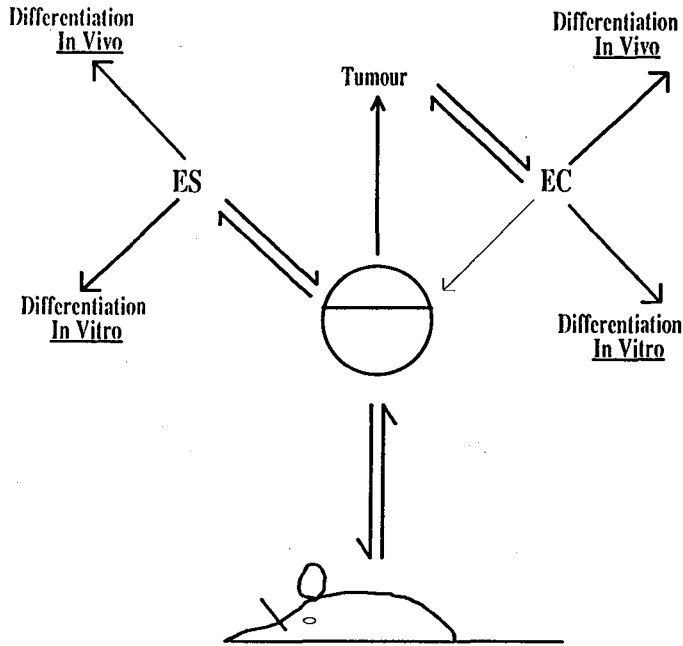
Fig. 1. The relationship between the normal mouse embryo, embryonal carcinoma (EC) cells and embryonic stem (ES) cells.

obtained from these surrogate systems can be used as a basis for the design of appropriate experiments to investigate the 'real' problem; the mammalian embryo itself.

Embryonal carcinoma and embryonic stem cells

Multipotential cell lines can be derived from early mammalian embryos by two methods (Fig. 1). The first (reviewed by Stevens, 1983) depends upon either the natural occurrence of germ cell tumours in specific mouse strains or the induction of teratocarcinoma tumours from the primitive ectoderm of embryos transplanted to ectopic sites. The continued growth of these tumours is due to the existence of a tumorigenic, pluripotential stem cell population, the embryonal carcinoma (EC) cells. EC cells can be taken into culture and grown as permanent cell lines where they may, under appropriate circumstances, differentiate into cell types which phenotypically and behaviourally resemble the derivatives of normal embryonic primitive ectoderm. Reintroduction of cultured EC cells into host mice yields teratocarcinoma tumours containing differentiated derivatives of EC cell origin. The second approach involves the derivation of pluripotential stem cell lines directly from embryos cultured *in vitro* (reviewed by Evans & Kaufman, 1983). These embryonic stem (ES) cells in many, but not all, respects resemble EC cells derived by means of tumour induction.

It is important to appreciate that extrapolation of results obtained from the

analysis of EC and ES cell lines to the normal embryo depends on the extent to which these cells can be shown to respond to normal developmental cues and signals. This can be tested by examining the developmental potential of the cells when re-incorporated into the normal embryo by blastocyst injection. From a large number of experiments of this type it is clear that EC cells are, in general, defective in their ability to differentiate correctly *in vivo* (Papaioannou & Rossant, 1983). Indeed, this inability of EC cells to differentiate efficiently *in vivo* may well be related to their tumour origin. ES cell lines, by contrast, can contribute to all tissues of the adult animal including the germ cell lineage (Bradley *et al.* 1984). ES cells are therefore without doubt genuine stem cells with pluripotential properties.

Thus EC cells, because of their embryonic origins, may be a valuable *source* of potential embryonic regulatory factors but are a less reliable guide to the possible *responses* of their normal embryonic counterparts. ES cells, on the other hand, appear to present a useful system for the identification of novel embryonic bioregulatory factors, since they are demonstrably capable of responding correctly to signals that exist in normal embryos.

EC cell-derived growth factors

Certain EC cell lines, in particular PC13 EC, have been used extensively for the analysis of growth factor action and expression *in vitro* (reviewed by Heath & Rees, 1985). They provide, subject to the important considerations outlined above, a favourable experimental system since they are blocked in their capacity to undergo spontaneous differentiation *in vivo* and *in vitro*. They may thus be propagated as homogeneous EC cell populations *in vitro*, and are consequently well suited to experimental study. Differentiation of these cells *in vitro* can be induced by exposure of the cells to retinoic acid (RA) yielding an apparently uniform cell type PC13 END, which resembles extra-embryonic mesoderm of the normal embryo. PC13 is therefore a simple cell system which can be switched from the stem cell into a single differentiation pathway by an externally applied signal.

This RA-induced differentiation is accompanied by significant changes in the control of cell proliferation, including loss of tumorigenic potential, acquisition of dependency upon exogenous growth factors for multiplication and a finite proliferative lifespan. However, the principal relevance of this system to the identification of embryonic polypeptide regulatory factors lies in the fact that the expression of endogenous growth regulatory factors is also developmentally regulated. Thus EC differentiation into END cells is accompanied by the expression of insulin-like growth factors (IGFs) which have the capacity to exhibit both paracrine and autocrine growth effects on END cells and their EC progenitors (Heath & Rees, 1985; Heath & Shi, 1986). This identification of IGF expression by an early embryo-derived cell type has led to an examination of IGF expression in early mammalian embryos where it was observed that IGF-like molecules were expressed by extra-embryonic mesoderm and amnion in early postimplantation development. Subsequent *in situ* hybridization studies using oligonucleotide probes have both confirmed and extended these observations, revealing a specific tissue-limited

expression of IGFs in the early postimplantation rodent embryo (Beck *et al.* 1987). This example clearly demonstrates the value of EC cells and their differentiated progeny as sources of embryonic polypeptide regulatory factors.

The ability to propagate PC13 EC cells as a homogeneous population *in vitro*, and the development of growth factor-free defined culture conditions (reviewed by Heath, 1987) permitted the analysis of bioregulatory factors secreted by EC stem cells. A potent, secreted, mitogenic activity was detected in serum-free PC13 EC cell conditioned medium whose expression ceased upon induction of differentiation (Isacke & Deller, 1983). The principal agent responsible for the mitogenic activity in PC13 EC cell conditioned medium was subsequently isolated and shown to be a single chain M_r 17 500 molecule termed ECDGF (embryonal carcinoma-derived growth factor, Heath & Isacke, 1984).

A key biochemical characteristic of ECDGF is its strong affinity for heparin (Heath, 1987; Van Veggel *et al.* 1987), a property which is shared with at least two other important biological regulatory factors, acidic (aFGF) (Giminez-Gallego *et al.* 1985) and basic fibroblast growth factor (bFGF) (Esch *et al.* 1985) first identified in adult neuronal tissues. This similarity between FGFs and ECDGF in heparin affinity and apparent molecular weight has led to a more detailed appraisal of the structural and functional relationship between ECDGF, aFGF and bFGF.

It is significant that ECDGF is a secreted factor, indeed its presence in conditioned medium provided the means for its identification. Both acid and basic fibroblast growth factor seem to be closely cell-associated in cultured cell lines and are not readily detected in conditioned medium (e.g. see Jaye *et al.* 1988). This may be due to the apparent absence of a secretory signal sequence in the primary acid or basic FGF translation product (Abraham *et al.* 1986).

Detailed analysis of the biochemical characteristics of ECDGF and bovine brain-derived acidic and basic FGF also reveals significant overall differences between the three factors; thus ECDGF elutes at comparable salt concentrations (1·3 M-NaCl) on heparin-derived HPLC affinity columns to aFGF, but both factors elute earlier than bFGF under the same conditions. In contrast ECDGF elutes at higher salt concentrations (0·6 M-NaCl, 10 mM-phosphate, pH 6·8) than either acid or basic FGF on mono-S cation-exchange matrices. ECDGF is also significantly more retarded when analysed by reverse phase chromatography than either acid or basic FGF. In summary, ECDGF can be physically separated from either acidic or basic FGF by a variety of different biochemical techniques.

That these three heparin-binding growth factors are distinct molecular species is further supported by three additional lines of evidence. First, the amino acid composition of ECDGF (data not shown) is significantly different from either acidic or basic FGF. Second an extensive series of antibodies generated against peptides derived from either the acidic or basic FGF amino acid sequence (Baird *et al.* 1986; unpublished observations of J. Slack and J. K. Heath) have been tested for cross-reaction with ECDGF by Western blotting techniques. With the exception of antibodies directed against residues 16–24 of basic FGF (but curiously not residues 15–30 which span the same region), no cross reaction has been observed. Finally,

distinct polypeptide maps are obtained from aFGF, bFGF and ECDGF when tryptic digests are analysed by microbore reverse phase HPLC.

All these findings therefore indicate that ECDGF is related to, but distinct from, the two 'prototype' heparin binding growth factors.

It has recently become apparent, however, that the 'FGF family' of growth factors is larger than had been hitherto suspected. A transforming oncogene *hst* has been isolated by standard NIH/3T3 transfection procedures from a human stomach tumour (Taira *et al.* 1987). An apparently identical gene *ks* has also recently been isolated from NIH/3T3 cells transfected with DNA taken from a Kaposi's sarcoma (Delli Bovi *et al.* 1987). The *ks/hst* gene encodes a functional mitogen with considerable sequence homology to the basic FGF prototype gene. A significant feature of *ks/hst* is the presence of a secretory signal sequence in the predicted primary translation product. Furthermore, this signal sequence appears to be functional since the product of the *ks/hst* gene can be shown to be secreted into the medium of cells when the gene is expressed under the control of a heterologous promotor (Delli-Bovi *et al.* 1987). Although expression of the *ks* gene in COS cells under the control of a heterologous promoter yields a secreted, functional *ks* protein of apparent M_r 24 000, the predicted primary sequence of the protein contains a number of paired basic amino acid residues which, if acting as substrates for proteolytic processing, could in principle produce a molecule of the same approximate apparent molecular mass as ECDGF isolated from EC cell-conditioned medium.

A second oncogene, initially identified by virtue of its activation in mammary tumours by retrovirus insertion, is *int-2*. DNA sequence analysis of the *int-2* gene also reveals significant sequence homology with the basic FGF prototype gene (Dickson & Peters, 1987). *int-2* transcripts are also expressed at low levels in F9 EC cells, although the level of transcription increases upon differentiation (Smith *et al.* 1988). In addition *int-2* transcripts display a strikingly localized pattern of expression in early mouse development when studied by *in situ* hybridization techniques (Wilkinson *et al.* 1988). It must be noted, however, that the *int-2* gene has yet to be shown to encode a functional mitogen and its exact relationship to other heparin-binding growth factors remains to be determined.

These findings prompt the speculation that these additional members of the FGF gene family, ECDGF, *ks/hst* and *int-2*, may be 'embryonic' forms of the FGF family and that heparin binding growth factors as a class have an important functional role in early development. This view finds support from the somewhat unexpected discovery that FGF-like factors have powerful biological actions upon developmental switches in early amphibian development.

Mesoderm induction

The differentiation of certain mesodermal tissues (such as muscle) in *Xenopus* appears to depend upon an inductive interaction between animal pole cells and the underlying vegetal endoderm, since isolated animal pole explants fail to generate

muscle and other mesodermal structures unless recombined with vegetal tissues. This inductive interaction appears to be one of the earliest intercellular signalling events in amphibian development, and plays a key role in subsequent morphogenesis of the embryo. It is very significant therefore that the inducing property of vegetal tissues can be, at least partially, reproduced with exogenously added natural (basic or acidic) FGF, ECDGF, or *ks/hst* protein synthesized *in vitro* (Slack *et al.* 1987; unpublished observations of G. Paterno, L. Gillespie, J. Slack and J. K. Heath). The case for FGF-like factors being the primary mesoderm-inducing agents *in vivo* is further strengthened by the finding that a *Xenopus* homologue of the mammalian basic FGF gene exists, and RNA transcripts hybridizing with this gene have been found to be expressed in embryos at stages where mesoderm induction is taking place (Kimelman & Kirschner, 1987). However, it is not at present clear whether *Xenopus* FGF itself or the equivalent of another member of the heparin binding growth factor family such as ECDGF or *ks/hst* is the primary agent of *Xenopus* mesoderm induction *in vivo*.

It is not at all apparent that mammalian development involves a developmental event similar to the induction of mesoderm in *Xenopus*. Nevertheless, this unexpected biological property of ECDGF, along with its derivation from embryo-derived EC cells, indicates that it may play a role in regulating early developmental decisions in the early mouse embryo.

This involvement of FGF-like factors in amphibian mesoderm induction has several important consequences for further studies of growth factor action in mammalian development. First, in general terms it reveals that agents initially identified by virtue of their mitogenic properties may also have profound effects on cell differentiation and morphogenesis in particular circumstances. Second, it shows that growth factors from mammalian sources exhibit considerable evolutionary conservation. This not only opens up the novel approach of analysing the possible biological function of growth factors in mammalian embryos by experiments in other species, but also conversely suggests that the homologues of other extracellular bioregulatory factors found in *Xenopus*, such as the XTC cell-derived mesoderm-inducing*factor (Smith, 1987) may have a biological role in mammalian development. Finally, the experimental accessibility of *Xenopus* embryos at early developmental stages compared to their mammalian counterparts suggests that they could have potential for the future identification of novel types of extracellular growth and differentiation regulatory factors from mammalian sources.

Embryonic stem cell differentiation

As discussed above, murine embryo-derived ES cells offer certain advantages over EC cells as an *in vitro* test system for the analysis of the response of early embryonic cells to growth and differentiation factors. However, an important drawback exists which has hitherto limited their use in the investigation of bioregulatory factor action. ES cells readily undergo spontaneous differentiation *in vitro* unless cultured in the presence of a live heterologous feeder cell layer. Although this is very

inconvenient from the experimental point of view, it may indicate something about the nature of the underlying mechanisms that control cell differentiation in this system. It appears that ES cells will enter a differentiation programme unless inhibited by a signal which can be provided by the feeder cells. This is in contrast to many teratocarcinoma-derived cell lines such as PC13, where entry into the differentiation programme is blocked unless an inducer, such as RA, is present.

It would obviously be of interest to understand the biochemical basis of the feeder cell effect on ES cell differentiation, since it would appear to affect mechanisms regulating a cellular decision to enter a differentiation programme which directly relates to a fundamental event in mammalian development, the differentiation of pluripotential primitive ectoderm. These mechanisms would, at least in part, appear to be the target for the changes underlying the loss of differentiation potential and acquisition of the malignant phenotype associated with EC cells. Indeed, rather as transformed fibroblasts lose the requirement for exogenous growth factor signals to maintain multiplication, it could be argued that EC cells have lost the requirement for exogenous differentiation inhibitory factors.

Isacke & Deller (1983) have demonstrated that the feeder cell effect, at least in the case of EC cells, is probably multifactorial, involving both cell contact-dependent and independent mechanisms. However, it is of some significance that the principal biological effect of feeder cells on ES cell differentiation can be reproduced by conditioned medium from various cell lines (Smith & Hooper, 1983; Koopman & Cotton, 1984). The medium from one cell line, Buffalo Rat Liver (BRL) has a particularly potent differentiation-inhibiting effect on multipotential EC and pluri-potential ES cells *in vitro* (Smith & Hooper, 1987). The effect of BRL conditioned medium is fully reversible, in that the cells can be maintained indefinitely in an undifferentiated state (in the absence of feeder cells) in its presence, but will rapidly differentiate at any time they are returned to normal unconditioned medium. Prolonged culture in BRL conditioned medium in the absence of feeder cells does not compromise the ability of ES cells to colonize the embryo in a chimaera or give rise to functional gametes. Hooper *et al.* (1987) have, for example, successfully used culture in BRL conditioned media to facilitate the isolation of hypoxanthine phosphoribosyl transferase-deficient ES cells which were used to generate ES cell derived germ line chimaeras transmitting HPRT-deficiency to progeny. The effect of BRL conditioned medium on ES cell differentiation is not reproduced by a variety of growth factors or growth modulators (such as IGF-II or TGFβ) known to be secreted by BRL cells (Smith & Hooper, 1987).

The biological action of BRL cell conditioned medium on ES cell differentiation depends upon a specific secreted polypeptide differentiation regulatory factor (termed differentiation inhibitory activity, DIA) which acts specifically to supress ES cell differentiation *in vitro*. Further analysis of the structure of DIA and its mechanism of action will undoubtedly have important consequences for our knowledge of the mechanisms controlling ES cell differentiation. Furthermore, BRL conditioned medium has already found use as a practical tool for the experimental manipulation of ES cell proliferation and differentiation *in vitro*.
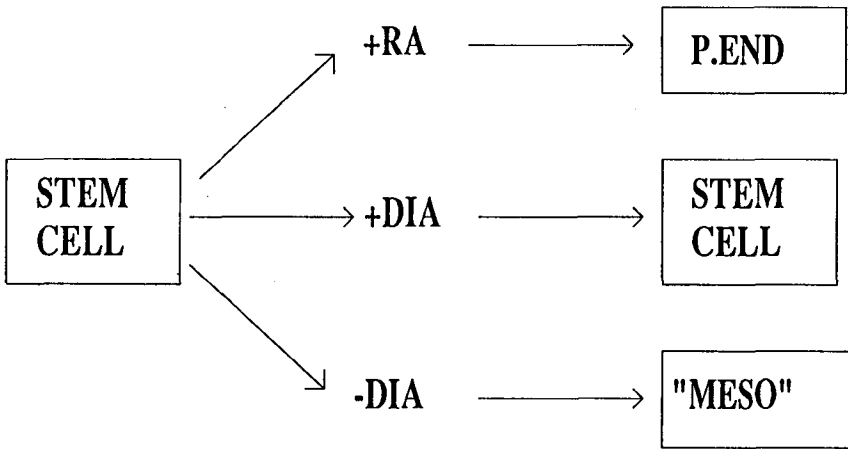
Fig. 2. The regulation of embryonic stem cell differentiation *in vitro* by differentiation inducers (RA) and differentiation inhibitors (DIA).

Mixing signals

It has emerged that the regulation of ES cell and EC cell differentiation depends upon exogenous signals; DIA suppresses differentiation whereas agents like RA induce differentiation. It is conceivable therefore, that the actual pattern of differentiation may depend upon the balance between differentiation-inducing and differentiation-suppressing extracellular signals. This can be investigated by exposing ES cells, cultured at clonal densities, to combinations of signals and observing the effects of these treatments on differentiation.

The results of this type of experiment are unexpected (summarized Fig. 2). In the presence of BRL medium as a source of DIA activity, CP1/86 ES cell differentiation is suppressed, and the cells are maintained in the ES cell state. Removal of DIA from cells grown in monolayer results in differentiation into a new cell type with mesodermal characteristics ('Meso'). Addition of RA to cells grown in the presence of DIA also results in the induction of differentiation, but the cell type formed is distinct from that formed by removal of DIA exhibiting morphological and molecular characteristics of parietal endoderm (Heath *et al.* 1988).

The conclusion from these experiments is that RA not only overrides the differentiation inhibitory action of DIA but also influences the differentiation programme that is chosen. It is likely, however, that at least some of this response to extracellular differentiation regulatory signals is more complex and depends upon both the target cell itself and spatial relationships between cells or other forms of local cellular interactions. First, ES cell lines such as CCE exhibit different patterns of differentiation in response to RA induction (not shown) from CP1/86 and, secondly, differentiation of ES cells in aggregates or high cell densities yields a broader spectrum of differentiated cell types than occurs in low density monolayer culture.

It is interesting to view these results in the light of concepts derived from the observation of stem cell behaviour in other systems (for a more extensive treatment

see Wolpert, 1989). It has often been argued that stem cells can be characterized by bifurcating cell divisions whereby a single stem cell divides to give rise to one differentiated daughter cell, and one daughter which retains the stem cell phenotype. In the case of ES cells, retention of the stem cell phenotype in these specific experimental conditions appears to be an 'all or none' property which is regulated by exogenous signals; both progeny of a single ES cell either enter a differentiation programme or do not. Clones comprising a mixture of ES cells and differentiated progeny are rarely, if ever, seen.

Conclusion

Although the identification and characterization of regulatory factor expression and action in early mammalian development seems like a technically intractable problem it will be apparent that some progress is beginning to be made. In particular, the exploitation of EC and ES cells as surrogate embryonic stem cells has facilitated biochemical characterization of at least some candidate regulatory factors. The finding that embryo-derived regulatory factors control developmental processes in amphibians has opened up a system with accessible embryonic stages for experimental analysis. Finally, the identification of secreted polypeptide factors which regulate ES cell differentiation *in vitro* suggests that cell differentiation, as well as cell proliferation, may be controlled by soluble polypeptide factors. The characterization of their mechanisms of action may prove illuminating.

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