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# The role of exogenous growth-promoting factors and their receptors in organogenesis

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**ABSTRACT The mechanisms involved in the regulation of early embryonic development are poorly understood. Certain growth promoting molecules are known to be produced within the embryo itself. It is clear, however, that at the early stages of embryonic development, many additional growth promoting factors have to be provided by the maternal system. Since the levels of factors such as epidermal growth factor and insulin in the maternal circulation are not linked with gestational age of the offspring, it is likely that regulation of receptors in the embryonic tissues may provide the key to the regulation of development. The expression of any receptor may depend on its synthetic rate, turnover or its distribution between the cell surface and intracellular pools. The study of the role of exogenous growth promoting molecules and receptor distribution and regulation for such growth factors, in particular insulin, insulin-like growth factor-I and epidermal growth factor, in embryos has been addressed using whole embryo culture, supported by anembryonic yolk sac culture and intravitelline injection of rat embryos.**

KEY WORDS: *embryo culture, yolk sac, microcannulation, growth factors*

## Growth factors in embryogenesis

During organogenesis the cells of the embryo may undergo proliferation, differentiation, migration and the process of apoptosis. These processes are controlled in general by extra- and intracellular signalling and growth factors may act as one such signal. Evidence exists that many maternally-derived growth factors may be involved in the regulation of embryonic growth (e.g. Rizzino, 1987; Mercola and Stiles, 1988). Since the levels of such factors in the maternal circulation do not correlate with different stages of gestation, the control mechanism for these molecules to regulate embryonic growth must be at the level of embryonic receptor expression. Most growth factors mediate their effects through specific receptors which often possess tyrosine kinase activity and promote a cascade of intracellular signals which usually lead to cell division. Some such molecules are regarded as competence factors, which permit cells to move from a quiescent state to enter the cell cycle e.g. PDGF and FGF. Others are progression factors which are concerned with the cell progressing through the stages of the cell cycle e.g. EGF, IGF-I (Basegra and Rubin, 1993).

The regulatory mechanisms involved in early mammalian embryonic development are in general poorly understood but recently many growth factors have been implicated in the control of growth and development during this period. Early studies showed that EGF could cause premature eyelid opening and incisor eruption (Cohen, 1962). More recently it has been shown that the development of the teeth in mice involves both EGF and FGF-4. In this study an interplay between the growth factors and the process of apoptosis (programmed cell death) has also been suggested (Vaahtokari *et al.*, 1996). EGF is also involved in fusion of the palatal shelves (Sharpe *et al.*, 1992) and binding sites for this molecule have been demonstrated in palatal mesenchyme and epithelium (Brunet *et al.*, 1993). It has therefore been suggested that perturbations of the interaction of the EGF receptor and this ligand, or TGF$\alpha$, may be important in the generation of cleft lip and palate (Sassani *et al.*, 1993). Serum IGF-I levels have been correlated with birth size and therefore linked to intrauterine growth retardation, as well as macrosomia in diabetic pregnancy (Lassarre *et al.*, 1991; Hill *et al.*, 1989). Transforming growth factor ß has been shown by gene knockout studies to play an important role in vasculogenesis and hematopoiesis, particularly in the rodent yolk sac (Dickson *et al.*, 1995). A highly specific role for several of the FGF family, in conjunction with various other signals, has been elucidated in pattern formation of the chick limb bud (as reviewed by Francis West and Tickle, 1996), although other workers have shown that IGF-I and insulin may also play an important role (Dealy and Kosher, 1996). In addition, the apoptosis necessary for the degeneration of interdigital material can be inhibited by local

*Abbreviations used in this paper:* BSA, bovine serum albumin; EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; PDGF, platelet derived growth factor.

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application of FGF-2 or FGF-4 in the embryonic chick (Macias *et al.*, 1996). Much of this is beyond the scope of this review, which will concentrate on the insulin-like factors and EGF.

It has been shown that certain growth promoting molecules are produced within the embryo itself (Gluckman, 1986; Adamson, 1983, 1990, 1993a; Mercola and Stiles, 1988), whilst others are postulated to be of maternal origin (Rizzino, 1987). For example, detailed studies using techniques such as Northern blotting and *in situ* hybridization have indicated that in the embryonic, fetal and neo-natal stages of development, mRNA for several growth factors is produced by developing tissues (e.g. insulin-like growth factor II: Beck *et al.*, 1987b; Han *et al.*, 1987; Senior *et al.*, 1990; Florance *et al.*, 1991; transforming growth factor α: Twardzik *et al.*, 1982; Huang *et al.*, 1996; transforming growth factor α and ß: Wilcox and Derynck, 1988). It is clear, however, that at the early stages of embryonic development, many additional growth promoting factors have to be provided by the maternal system.

Recent studies using gene knockout as a tool have indicated that in many cases the lack of expression of a single growth factor gene has relatively little effect on growth and survival, in a few cases causing some small abnormalities only. This has led to the theory that many functions of the growth factor families are subject to redundancy and suggested that just because a gene is highly conserved and abundantly expressed this does not mean it is essential (Shastry, 1994). This gene redundancy may also extend to the ability of the maternal system to compensate for deficits in the embryonic make-up, or indicate that certain factors are normally supplied exogenously *in utero*.

## Embryo culture: a tool to study factors affecting organogenesis

The development of techniques permitting the use of rodent embryos grown in culture during the period of organogenesis (New, 1978) opened up novel areas for experimental embryology. It has been rendered particularly useful by the ability to accurately quantify the extent of embryonic growth and differentiation (Brown and Fabro, 1981; Picard and Van Maele Fabry, 1987). Thus, in addition, it had the potential for use in experiments to elucidate the mechanism of action of various teratogens (Kitchen *et al.*, 1986; Cicurel and Schmid, 1988). The technique has been used to investigate the effects of ethanol (Brown *et al.*, 1979; Clode *et al.*, 1987), retinoids (Steele *et al.*, 1987; Klug *et al.*, 1989), folate deficiency (Miller *et al.*, 1989) and drugs such as valproate (Anwar *et al.*, 1990) and aspirin (McGarrity *et al.*, 1981). Because many of the effects seen in *in vivo* teratology can be, at least to some extent, mimicked in whole embryo culture, it has been suggested that this technique may be of use as a predictor of teratogenicity and may fulfil a role as an *in vitro* screen (as reviewed by Brown and Fabro, 1982; Steele and Copping, 1990).

It has been used to study the factors which may cause the increased malformations seen in diabetes (Cockroft, 1984; Sadler, 1980; Travers *et al.*, 1989, 1992). Further, it has been used in conjunction with other techniques to investigate the teratogenicity of thalidomide (Cumberland and Pratten, 1995). Also, its potential for the identification of human teratogens and embryopathic factors has been investigated by the use of human serum as a culture medium (Chatot *et al.*, 1980; Anwar and Beck, 1988; Gulamhusein *et al.*, 1990). It has been shown that serum from women with increased risk of miscarriage (Klein *et al.*, 1983; Anwar *et al.*, 1987) or those carrying a neural tube defect-affected fetus (Anwar *et al.*, 1989) are frequently unable to support normal rat embryonic growth in culture.

More usefully the technique has been employed in endeavors to elucidate the means by which normal growth and differentiation may be perturbed. In particular the mechanism of action of toxic molecules found either in the environment or administered as therapeutic agents. Thus in recent years the role of HOX genes and apoptosis in mediating such effects at the cellular level have been studied. Also, perturbations in the synthetic functions of the yolk sac and embryo are thought to be important.

## The role of low molecular weight molecules in organogenesis

In the past, a large volume of data has accumulated concerning the nutritional requirements of developing embryos (e.g. Cockroft, 1984, 1988). Attempts have been made to grow embryos in defined media, but it has been found that serum is an essential part of culture media for normal embryonic growth and differentiation *in vitro*. Evidence is available indicating that whilst extremely low molecular weight material is not specifically required by embryos in culture (Klein *et al.*, 1978; Gulamhusein *et al.*, 1990), substances in the molecular weight range 5-30 kDa are essential to normal growth and development (Andrews *et al.*, 1987; Gulamhusein *et al.*, 1990). Other data indicate that during embryo culture certain biopolymers are specifically depleted from the culture serum (Sanyal, 1980). It has been shown that 'exhausted' serum can be replenished by supplementation with whole rat serum (Pratten *et al.*, 1988). In addition partial restoration of growth has been observed in the presence of physiological concentrations of EGF, insulin and transferrin.

Several other growth promoting factors have also been indicated for embryonic growth *in vitro*. When human serum is used as a culture medium for rat post-implantation conceptuses, the embryos that result are frequently dysmorphic and anaemic. Supplementation with 5-10% rat serum permits embryonic growth that matches that seen with 100% rat serum (Anwar and Beck, 1988). When rat serum fractions are used as a supplement it is found that high molecular weight (>30 kDa) material is required to alleviate the anemia, whereas the low molecular weight (<30 kDa) fraction improves growth and differentiation (Gulamhusein *et al.*, 1990). Further studies have shown that rat transferrin is a species-specific factor for rat embryos (Cumberland and Pratten, 1993).

Recently, the rat conceptus has been used to study the timing of expression of certain growth promoting molecules (Beck *et al.*, 1987b). In addition the role of maternally derived factors such as growth factors, hormones and immune factors has been studied. Since the levels of factors such as epidermal growth factor and insulin in the maternal circulation are not linked with gestational age of the offspring, it is likely that regulation of receptors in the embryonic tissues may provide the key to the regulation of development. The expression of any receptor may depend on its synthetic rate, turnover or its distribution between the cell surface and intracellular pools. The study of receptor distribution and regulation for such growth factors in embryos may thus hold the key to some of these questions.

## Functions of the visceral yolk sac

The role of the visceral yolk sac in the maintenance of normal embryonic development has been investigated extensively. Because the yolk sac and the amnion are the only extraembryonic tissues required to maintain the growth and differentiation of rodent conceptuses during the period of organogenesis in culture, the functions of the yolk sac have been the subject of many studies. Thus, it has been shown that the yolk sac has very active endocytic mechanisms, which are involved in the nutrition of the embryo. The yolk sac is known to capture exogenous proteins at a high rate by adsorptive endocytosis (Williams *et al.*, 1975a,b; Livesey and Williams, 1979), digest them in the lysosomal system, and transport the amino acid breakdown products to the embryonic face. The incorporation into embryonic protein of radiolabeled amino acids derived from exogenous proteins has been demonstrated using the whole conceptus in culture (Freeman *et al.*, 1981). The yolk sac is the site of synthesis of various macromolecules, including alpha-fetoprotein (Abelev, 1971) and insulin-like growth factor I (Beck *et al.*, 1987b). In addition, it can capture and transport various macromolecules, such as transferrin (Huxham and Beck, 1984, 1985; McArdle and Priscott, 1984; Cumberland and Pratten 1993) and immunoglobulin G (Huxham and Beck, 1981; Weisbecker *et al.*, 1983). There is also evidence that the yolk sac serves as a liver for the conceptus prior to the establishment of the fetal liver. It possesses many liver-like functions, including inducible cytochrome P450 (Rowlands *et al.*, 1988; Simpson *et al.*, 1996).

Because of the complex role of the yolk sac, and in particular its capacity to transport specific molecules as well as digest exogenous material, it is not clear whether a growth factor is likely to interact with the yolk sac receptors in the usual way, or interact with a receptor that permits transport of the molecule to the embryo intact. Thus it is possible that the growth factor binds to a growth factor receptor at the surface, causes autophosphorylation of the tyrosine residues and sets off a cascade of intracellular signals. These signals could result in the yolk sac expanding due to the triggering of cell division. Alternatively, the effect could be on the expression of various genes in the yolk sac which might result in altered uptake or transport functions. This could then indirectly have an effect on embryonic growth.

On the other hand, because of the known transport routes which avoid the lysosomal compartment available in the yolk sac for certain specific molecules, it is conceivable that the growth factor receptors on the yolk sac are principally not for the transduction of signals at the yolk sac surface but for transport of these factors to the embryo intact. Such a routing would require a sorting mechanism within the endosomal compartment of the yolk sac cell so that the receptor-ligand complex could be directed to the base of the cell and thus into the vitelline circulation and to the embryo.

Many studies have been undertaken concerning the uptake and processing of macromolecules by the rat visceral yolk sac in

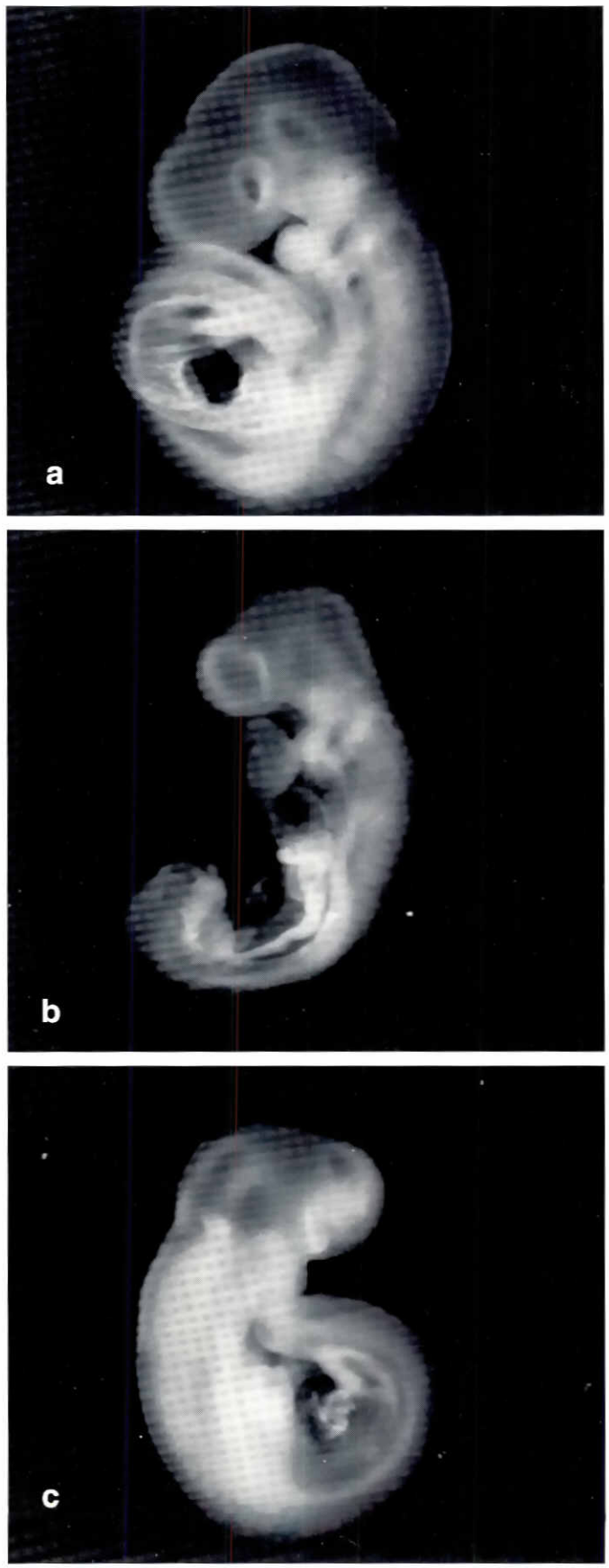


**Fig. 1. Effect of EGF on growth of rat embryos cultured in depleted sera *in vitro*.** *Conceptuses were explanted on gestational day 9 and cultured in serum which had been subjected to ultrafiltration to remove material below 30 kDa molecular weight, in the presence or absence of EGF at a concentration of 8 ng/ml. Embryos were harvested after 48 h culture by the method of New;* **(a)** *control embryo cultured in whole rat serum;* **(b)** *embryo cultured in 30 K retenate;* **(c)** *embryo cultured in 30 K retenate plus 8 ng/ml EGF.*

culture. The vast majority of these have been performed at 17.5 days of gestation using yolk sacs obtained direct from the mother and cultured for up to 24 h in medium 199/calf serum (Williams *et al.*, 1975a,b; Roberts *et al.*, 1977; Duncan and Lloyd, 1978). Included in the medium have been substrates captured by fluid-phase pinocytosis ($^{125}$I-labeled polyvinylpyrrolidone, sucrose, dextran), by adsorptive pinocytosis (e.g. denatured bovine serum albumin, lactate dehydrogenase, various polymers) and receptor mediated pinocytosis (immunoglobulin G, growth factors, transferrin). This system is extremely reliable, giving very reproducible results, such that the rates and mechanism of uptake and processing can be readily compared for different substrates (reviewed by Duncan and Pratten, 1985).

One limitation of this method is that although the cells are polar *in vivo*, having a maternal and an embryonic face, when they are cultured as an open vesicle this orientation is lost. Thus, it is impossible to use this system to study vectorial transport of molecules from the mother to fetus. Several attempts have been made to create an artificial polarity using two-chambered culture vessels which trapped yolk sacs between the two sides of the vessel. Such methodology was found to have enormous problems of leakage and cell death. More recently attempts have been made to culture endodermal yolk sac cells on artificial membranes such that the cells are confluent and form tight junctions (Wild, personal communication). The vectorial transport of specific substrates such as IgG can be studied using this method. However, this system has some drawbacks in terms of non-specific transport, and the cells are not in their natural situation.

In the early 1980's a system was developed which permitted the prolonged culture of rat conceptuses from day 9.5 of gestation to up to 18.5 days. During this extended culture, the embryo was found to die, but the visceral yolk sac continued to grow until it was around 2 cm in diameter and contained over 1 ml of extraembryonic coelomic fluid. Studies with Lanthanum nitrate indicated the presence of tight junctions, and this therefore provided an excellent system for uptake studies across a polar endodermal layer (Dunton *et al.*, 1986; Pratten *et al.*, 1987). Thus it was found that the rate of uptake of radiolabeled polyvinylpyrrolidone, a marker for fluid-phase non-adsorptive pinocytosis, was identical to that observed in yolk sacs explanted on day 17.5. In addition studies were performed which demonstrated the uptake and degradation of formaldehyde-denatured $^{125}$I-labeled bovine serum albumin, and in particular showed the vectorial transport of digestion products to the embryonic face of the yolk sac (Dunton *et al.*, 1988). A further sophistication of this technique has been the removal of the embryonic pole of the conceptus prior to culture. After culture the yolk sac was found to expand and grow normally, such that it was of similar size to a conceptus with the embryo present during the first 48 h of culture, but could be maintained in culture for up to 10 days. Many experiments have been undertaken which have indicated that this system is organotypic and shows a similar enzymatic developmental profile to that observed *in vivo*. This culture system for anembryonic yolk sacs was a clear improvement on the earlier method, since any effect of the remains of the embryo was removed. It was therefore possible to study the uptake and processing of substrates by the yolk sac, and, more importantly, study the transport of intact proteins to the embryonic face of the yolk sac cells.

## Is maternally derived insulin required for embryogenesis?

There is evidence for the receptors for insulin early post-implantation embryos (Smith *et al.*, 1987; Baker *et al.*, 1993), but very little evidence exists for synthesis of insulin either by immunocytochemistry (Travers, 1989) or *in situ* hybridization (Beck *et al.*, 1987b). Early studies had indicated that the growth promoting properties of serum depleted by repeated culture of rat conceptuses could be partially restored by the addition of insulin at physiological concentrations (Pratten *et al.*, 1988). However, Travers and colleagues (1989) have shown an absolute require-

TABLE 1

**EFFECT OF EGF ON THE DEVELOPMENT OF RAT CONCEPTUSES IN RETENATE CULTURE**

| culture medium | n | morphological score | yolk sac diameter (mm) | crown rump length (mm) | somite number | embryonic protein content (μg) | yolk sac protein content (μg) |
|---|---|---|---|---|---|---|---|
| whole rat serum | 44 | 58.0** (55.1-60.5) | 3.4** (3.1-3.6) | 3.0** (2.8-3.2) | 23.0** (21.3-24.0) | 197.0** (140.8-226.8) | 193.0** (151.5-259.3) |
| retenate | 42 | 41.8 (35.8-44.0) | 2.8 (2.6-2.9) | 2.5 (2.1-2.6) | 14.0 (11.8-18.0) | 115.0 (75.5-142.3) | 86.5 (64.3-136.8) |
| 2 ng/ml EGF | 15 | 41.0 (35.0-44.0) | 2.8 (2.6-2.9) | 2.5 (2.4-2.7) | 13.0 (11.0-15.0) | 169.0** (134.0-182.0) | 149.0** (128.0-182.0) |
| 4 ng/ml EGF | 19 | 41.0 (34.0-50.0) | 2.7 (2.5-2.8) | 2.5 (2.3-2.8) | 13.0 (10.0-16.0) | 99.0 (91.0-126.0) | 99.0 (76.0-111.0) |
| 8 ng/ml EGF | 19 | 51.0** (45.5-55.5) | 3.1** (2.9-3.2) | 2.7** (2.5-2.9) | 21.0** (18.0-22.0) | 120.0 (83.0-149.0) | 131.0* (114.0-162.0) |
| 16 ng/ml EGF | 17 | 35.0 (29.3-44.5) | 2.5* (2.3-2.8) | 2.4 (2.0-2.6) | 9.0* (4.0-16.0) | 86.0 (71.0-108.0) | 63.0 (48.5-85.0) |
| 32 ng/ml EGF | 16 | 42.8 (32.3-48.0) | 2.9* (2.8-3.0) | 2.6* (2.5-2.78) | 15.0 (11.0-19.0) | 144.5 (108.0-169.5) | 120.5 (81,8-166.8) |

n, number of conceptuses; *significantly different from retenate at $p<0.05$; **significantly different from retenate at $p<0.001$. Results are expressed as medians with ranges in parentheses.

TABLE 2

**EFFECT OF LONG EGF ON THE DEVELOPMENT OF RAT CONCEPTUSES IN RETENATE CULTURE**

| culture medium | n | morphological score | yolk sac diameter (mm) | crown rump length (mm) | somite number | embryonic protein content (μg) | yolk sac protein content (μg) |
|---|---|---|---|---|---|---|---|
| whole rat serum | 19 | 57.5** (53.5-59.0) | 3.4** (3.2-3.9) | 3.1** (2.8-3.4) | 23.0** (21.0-23.0) | 143.0** (127.0-160.0) | 144.0** (135.0-157.0) |
| retenate | 20 | 37.0 (31.5-39.4) | 2.9 (2.8-3.2) | 2.6 (2.5-2.9) | 12.5 (11.3-14.8) | 101.5 (78.5-119.8) | 107.0 (101.3-122.3) |
| 2 ng/ml long EGF | 13 | 39.0 (32.3-41.5) | 3.0 (2.8-3.1) | 2.8 (2.6-2.9) | 14.0 (11.5-16.5) | 102.0 (84.0-116.5) | 110.0 (90.5-115.5) |
| 4 ng/ml long EGF | 13 | 42.0* (36.5-43.8) | 3.0 (2.9-3.1) | 2.8* (2.6-3.0) | 16.0** (13.5-17.5) | 118.0* (97.0-137.5) | 117.0 (110.0-130.5) |
| 8 ng/ml long EGF | 15 | 39.5* (38.0-42.5) | 3.1* (2.9-3.5) | 3.0* (2.6-3.3) | 15.0* (14.0-16.0) | 115.0 (90.0-131.0) | 122.0 (101.0-136.0) |
| 16 ng/ml long EGF | 12 | 40.2* (37.1-41.0) | 3.1* (2.9-3.4) | 2.85* (2.7-3.7) | 15.0* (14.3-16.0) | 117.5 (100.3-128.8) | 120.0 (94.0-132.2) |
| 32 ng/ml long EGF | 12 | 37.0 (34.0-40.1) | 2.9 (2.7-3.1) | 2.8 (2.5-3.0) | 13.0 (12.0-15.5) | 110.5 (93.5-123.5) | 112.5 (101.3-131.3) |

n, number of conceptuses; *significantly different from retenate at $p<0.05$; **significantly different from retenate at $p<0.001$. Results are expressed as medians with ranges in parentheses.

TABLE 3

**EFFECT OF INHIBITION OF THE EGF RECEPTOR ON EMBRYONIC GROWTH AND DEVELOPMENT**

| culture medium | n | morphological score | yolk sac diameter (mm) | crown rump length (mm) | somite number | embryonic protein content (μg) | yolk sac protein content (μg) |
|---|---|---|---|---|---|---|---|
| whole rat serum | 13 | 59.0 (58.0-60.0) | 3.6 (3.1-4.0) | 3.2 (2.8-3.6) | 24.0 (22.0-25.0) | 160.0 (127.0-219.0) | 164.0 (144.5-167.5) |
| DMSO control | 15 | 59.0 (52.0-60.5) | 3.3 (3.3-3.8) | 3.0 (2.8-3.2) | 23.0 (21.0-25.0) | 164.0 (132.0-190.0) | 130.0 (115.0-181.0) |
| 25 μM tyrphostin 47 | 9 | 47.0* (41.3-49.8) | 2.8* (2.6-3.0) | 2.5* (2.4-2.7) | 20.0* (19.5-22.0) | 97.0* (77.0-130.5) | 86.0** (75.5-107.5) |
| 50 μM tyrphostin 47 | 10 | 49.3* (40.6-51.9) | 2.7* (2.2-3.3) | 2.5* (2.3-2.9) | 20.5* (17.5-22.5) | 117.0* (60.0-136.8) | 99.0* (72.5-124.5) |
| 100 μM tyrphostin 47 | 15 | 44.0** (33.0-48.0) | 2.6** (2.0-2.8) | 2.3** (1.9-2.5) | 19.0** (11.0-21.0) | 73.0** (51.0-128.0) | 84.0** (68.0-97.0) |
| 100 μM tyrphostin 1 | 9 | 57.5 (52.8-58.3) | 3.7 (3.3-3.8) | 3.0 (2.9-3.3) | 23.0 (21.5-24.0) | 155.0 (116.5-163.0) | 144.0 (117.0-167.5) |

n, number of conceptuses; *significantly different from DMSO control at $p<0.05$; **significantly different from DMSO control at $p<0.001$. Results are expressed as medians with ranges in parentheses.

ment for exogenous insulin by pre-somite 9.5 day rat embryos grown in culture. Using affinity chromatography to deplete insulin specifically from the culture serum to levels less than 0.5 ng/ml, as measured by radio-immune assay, it has been shown that such serum cannot support normal embryonic growth. Addition of 10 ng/ml exogenous porcine insulin can restore normal growth promoting properties to the serum. Also, when guinea-pig serum was used as a culture medium for post-implantation rat embryo culture, the embryos were found to be dysmorphogenic, with over 85% of embryos abnormal, compared to no abnormalities recorded when embryos were cultured in rat serum. This was thought to be caused by the difference in structure and potency of guinea pig insulin compared to rat insulin. Indeed when insulin was added at physiological concentrations to guinea pig serum it showed an improved ability to support normal growth of rat embryos (Travers *et al.*, 1992).

## The role of insulin like-growth factors and their receptors

Further studies were undertaken to investigate the uptake and processing of insulin and IGF-I, factors which had both been previously shown to be important when presented exogenously to rat conceptuses in culture (Travers *et al.*, 1992). These two ligands are very similar structurally, and both possess receptors which are dimers with tyrosine kinase activity. Although either ligand can interact with either receptor, they possess much greater affinity for their own receptor. In the case of the growing rat conceptus, it is not clear whether both receptors, or only one is involved in uptake and processing of these ligands. Uptake of insulin appeared to differ from that of IGF-I when 17.5 day visceral yolk sacs taken directly from the mother were employed and given radiolabeled ligands (Cowley and Pratten, 1992). Also it has been found that the two molecules seem to be located in different vesicles and those

TABLE 4

**EFFECT OF INTRAVITELLINE INJECTION OF TYRPHOSTINS ON EMBRYONIC GROWTH AND DEVELOPMENT**

| culture medium | n | morphological score | yolk sac diameter (mm) | crown rump length (mm) | somite number | embryonic protein content (μg) | yolk sac protein content (μg) |
|---|---|---|---|---|---|---|---|
| DMSO control | 25 | 24.0 (21.0-25.5) | 4.8 (4.5-4.9) | 4.3 (4.0-4.5) | 30.0 (29.0-31.0) | 280.0 (255.0-307.0) | 194.0 (178.0-208.5) |
| 50 μM tyrphostin 47 | 17 | 18.0** (17.0-21.0) | 4.2* (4.1-4.7) | 3.9* (3.8-4.1) | 27.0** (25.5-28.0) | 289.0 (274.0-327.5) | 189.0 (180.5-223.5) |
| 100 μM tyrphostin 47 | 16 | 19.0** (16.3-20.0) | 4.4* (4.0-4.7) | 4.0* (3.8-4.2) | 25.0** (25.0-27.8) | 282.0 (245.5-308.0) | 202.0 (184.3-216.3) |
| 200 μM tyrphostin 47 | 17 | 19.0** (16.0-21.5) | 4.5 (4.2-4.9) | 4.1 (4.0-4.4) | 26.0** (25.5-29.0) | 294.0 (257.0-305.5) | 192.0 (184.0-216.0) |
| 200 μM tyrphostin 1 | 13 | 19.0* (16.0-25.0) | 4.6 (4.1-4.8) | 4.2 (3.8-4.6) | 28.0* (27.5-30.0) | 273.0 (208.5-293.0) | 194.0 (208.5-293.0) |

n, number of conceptuses; *significantly different from DMSO control at $P<0.05$; **significantly different from DMSO control at $P<0.001$. Results are expressed as medians with ranges in parentheses.

containing IGF-I only were found deeper in the cell (Cowley and Pratten, 1996). Anembryonic yolk sac culture, where the yolk sac is cultured as a closed vesicle, which permits the study of vectorial transport of molecules (Dunton *et al.*, 1988) has confirmed this. When the two ligands, insulin being tagged with fluorescein and IGF-I being tagged with rhodamine, were presented simultaneously the vesicles containing IGF-I were the only ones which were found towards the base of the cell and therefore able to be presented to the embryonic face of the yolk sac. Thus material could be delivered into the vitelline circulation for transfer to the embryonic circulation. This therefore may suggest that the two molecules, although similar, are actually handled differently and that whereas insulin is processed via the endosomal/lysosomal system, IGF-I may be transported to the embryo intact.

A role for the insulin-like growth factors has been suggested during palatal closure (Ferguson *et al.*, 1992). Gene knockout studies of mice carrying null mutations for the insulin-like growth factors and the type 1 and type 2 receptor have shown that the type 1 receptor is of greatest importance for maintenance of growth of the embryo, whereas type 2 receptors or an additional unknown receptor, may play a role in the maintenance of the placenta (Baker *et al.*, 1993). Null mutations of the IGF-I gene show a 60% growth retardation, and some of these die shortly after birth. When the type-1 receptor is deleted, the pups are only 45% of normal size and die of respiratory failure at birth, exhibiting general delays in muscle and skeletal development (Liu *et al.*, 1993).

## The role of epidermal growth factor and its receptor

In the case of epidermal growth factor, the receptor has been identified in embryonic tissues (Nexo *et al.*, 1980; Hortsch *et al.*, 1983; Adamson and Meek, 1984; Paria and Dey, 1990) but evidence indicates that there is no embryonic synthesis of the molecule until day 19 in the rat (Popliker *et al.*, 1987; Raaberg *et al.*, 1988). However, EGF is known to be produced by the decidual cells immediately surrounding the growing conceptus in the mouse (Huet-Hudson *et al.*, 1990) The receptor for EGF is known to be expressed even at the pre-implantation stage of mouse development (Adamson *et al.*, 1981; Adamson, 1993b). Targeted disruption of the EGF receptor has indicated an important role for this molecule, particularly in mid-gestation in the mouse (Sibilia and Wagner, 1995; Threadgill *et al.*, 1995). Studies have indicated a role for epidermal growth factor as a supplement to media depleted of low molecular weight material by stringent dialysis or ultrafiltration (Andrews *et al.*, 1987). Such sera may be prepared by the use of immersible filters with a cut off of 10 or 30 kDa, such that serum may be split into two fractions, filtrate, containing low molecular weight material and retenate, containing high molecular weight material. Experiments have shown that neither of these fractions can support normal embryonic growth in culture but when these two fractions are added back together, normal growth is restored. When EGF is added to such sera, a dose-dependent improvement in growth is observed. Figure 1 shows rat embryos cultured from day 9 to day 11 in whole rat serum, retenate and retenate to which 10 ng/ml EGF have been added. As can be seen there is a partial restoration of growth promoting properties which suggests that exogenous EGF may have a role for embryogenesis.

Recent work in this laboratory has confirmed a role for EGF (Table 1) and by also making use of the more stable molecule 'long EGF', which has an amino terminal extension of 53 amino acids, has shown (Table 2) that growth of embryos can be promoted by molecules of the EGF family. When added at concentrations between 4 and 16 ng/ml, long EGF enhances the growth and development of embryos grown in serum depleted of low molecular weight factors, in this case using centrifugal ultrafiltration. Furthermore, the specific EGF receptor signal transduction inhibitor Tyrphostin 47 (Gazit *et al.*, 1989; Levitzki and Gilon, 1991; Levitzki, 1992) can inhibit embryonic growth when administered in culture (Table 3), whereas Tyrphostin 1 which has a low affinity for the tyrosine kinase of the EGF receptor was without effect (Tebbs *et al.*, 1997). It has also been shown that gene knockout of TGF, the embryonically produced EGF analog, does not completely prevent development (Ferguson, personal communication) probably because of the capture of exogenous EGF, at the time when the embryo would normally be self-sufficient. Mice with a null mutation of the TGF gene have abnormalities of the skin and its appendages, in particular having curly whiskers and wavy hair (Luetteke *et al.*, 1993; Mann *et al.*, 1993). The similarity between this phenotype and that of the mutant waved-1 mouse have led to the conclusion that this is a mutation of the EGF (TGFα) receptor gene (Luetteke *et al.*, 1994).

Additionally, epidermal growth factor has been shown to be avidly captured by the visceral yolk sac of the 11.5 day conceptus in culture (Andrews *et al.*, 1987) and also by anembryonic yolk sacs grown in culture.

## Is the site of EGFR action at the yolk sac or embryo?

In the rat conceptus, the material processed by the visceral yolk sac is thought to be routed to the embryo by means of the vitelline circulation. The vitelline vessels lie immediately below the endodermal cells of the yolk sac. Because the yolk sac is so metabolically active, and tends to digest or modify most substrates presented to it, it has been difficult to study the way in which the embryo itself handles any macromolecules that it encounters. A system was therefore developed (Beck *et al.*, 1987a; Mensah-Brown *et al.*, 1987) which permitted cannulation of the vitelline circulation of rat conceptuses on day 11 of gestation using glass cannulae pulled to an outside diameter of 10 μm. In order to prevent the loss of blood vessel contents, the vessel was subsequently sealed both proximal and distal to the injection site using diathermy. In a detailed study, it was shown that there was no significant difference between embryos grown *in vitro* and those explanted later in the day. Injection did not have any effect on subsequent growth and development of embryos in culture unless the injection volume exceeded 2 μl (Mensah Brown *et al.*, 1989).

This method has more recently undergone a great deal of refinement in order to make it possible to microcannulate the circulation without the necessity to use the damaging diathermy to occlude vessels. In this way it is possible to extend the culture period to at least 30 h post-injection. This has been achieved by using cannulae of around 1.2 μm diameter and a microinjection pump to deliver nanolitre quantities of fluid into the vitelline circulation (Cumberland *et al.*, 1992a; Cumberland and Pratten, preliminary patent). It is also possible to cannulate the heart on day 10.5 using this technique. The technique has been used extensively to deliver molecules which are either known to be teratogenic or non-teratogenic, including a blind trial of over 30 compounds (Cumberland *et al.*, 1992b, 1994). It has also been used in conjunction with whole embryo culture in rat and human serum to investigate the teratogenicity of thalidomide and its analogues (Cumberland and Pratten, 1995).

It has been possible to use this refinement of the original technique, because of the extended culture period, to look at the direct effects on the embryo of various inhibitors of growth factor receptor function. Thus, experiments have been undertaken where the EGF receptor tyrosine kinase inhibitor, Tyrphostin 47, has been administered to embryos on day 11 and their growth and differentiation evaluated on day 12 of gestation (Table 4, data described in Tebbs *et al.*, 1997). A dose dependent decrease in developmental score was observed with Tyrphostin 47, whereas Tyrphostin 1, a molecule with minimal inhibitory effects on the EGF receptor, was in general without effect even at high dose. Further preliminary studies used this technique to administer an antibody inhibitor of PDGF function to 11 day embryos and in this case, inhibition was observed with polyclonal goat anti-PDGF antibody (Promega), whereas non-specific goat IgG was without effect (Pratten, Khan and Cumberland, unpublished).

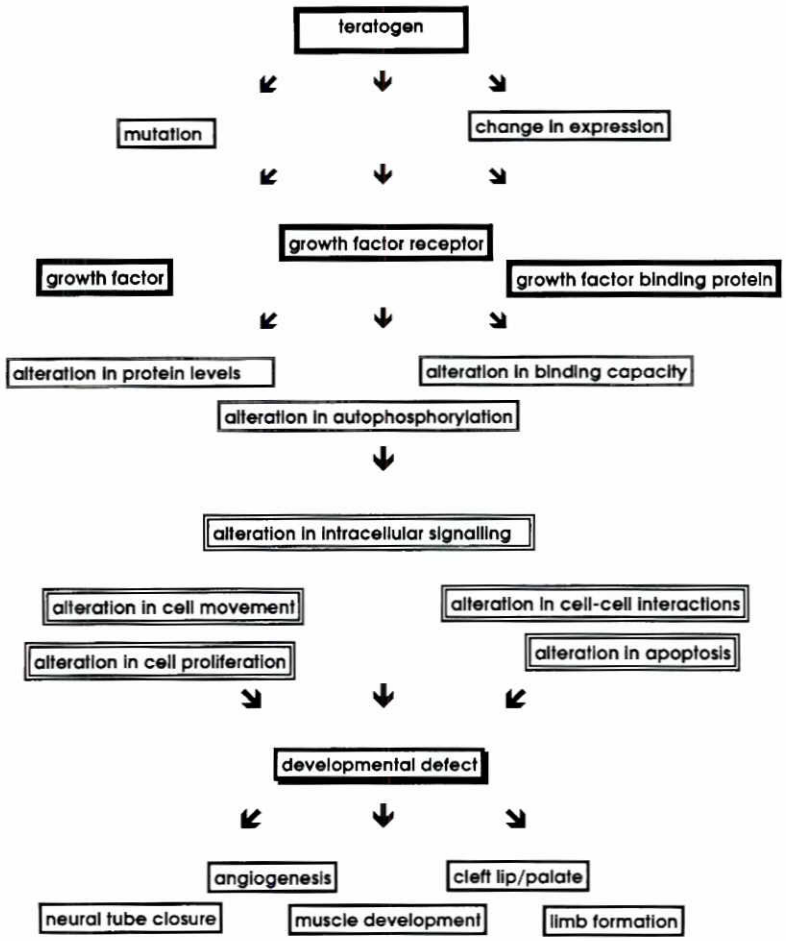


**Fig. 2. Some potential mechanisms whereby disruptions to growth factor signaling pathways may cause developmental pathology.**

## Could perturbations in growth factor function be a mechanism for teratogenesis?

A role for growth factors has been suggested in the generation of small for date babies caused by intrauterine growth retardation. It would therefore be of interest to compare the uptake and processing of growth factors, as well as receptor distributions, in normal embryogenesis and embryos under various types of stress, such as nutritional deficit or in the presence of teratogens such as retinoic acid, as has been seen in the palate (Abbott and Pratt, 1991) and placenta (Roulier *et al.*, 1994), or ethanol, valproate etc. The potential role for growth factor perturbations is outlined in Figure 2.

## Conclusion

The development of culture systems for rat conceptuses by New and his colleagues (reviewed in New, 1978) was a major breakthrough in experimental embryology and *in vitro* teratology. Thus it has been used to evaluate the role of growth factors in embryonic growth (Pratten *et al.*, 1988; Gulamhusein *et al.*, 1990; Tebbs *et al.*,

1997) and the effects of diabetic pregnancy (Sadler, 1980; Travers *et al.*, 1989, 1992), as well as the effects of alcohol consumption (Clode *et al.*, 1987) or folate deficiency (Miller *et al.*, 1989). It has been suggested by several authors that the whole-embryo culture system could be used as a predictor of embryotoxicity and teratogenicity (e.g. Kitchen *et al.*, 1986; Cicurel and Schmid, 1988). It certainly has a role to play in the investigation of the molecular mechanisms involved in the control of embryonic growth and differentiation and how these may be perturbed to generate abnormal fetuses. In recent years the development of other techniques based on embryo culture, such as anembryonic yolk sac culture and intravitelline injection, offer complimentary ways of studying the role of the yolk sac in isolation from the embryo and a means of direct delivery of materials to the embryo which by-passes the metabolic and barrier effects of the yolk sac. These methods should therefore provide the means to further elucidate the mechanisms of action of factors which promote or inhibit embryonic growth and development.

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