Growth hormone, insulin-like growth factor I and cell proliferation in the mouse blastocyst

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The role of growth hormone (GH) in embryonic growth is controversial, yet preimplantation embryos express GH, insulin-like growth factor I (IGF-I) and their receptors. In this study, addition of bovine GH doubled the proportion of two-cell embryos forming blastocysts and increased by about 25% the number of cells in those blastocysts with a concentration–response curve showing maximal activity at 1 pg bovine GH ml⁻¹, with decreasing activity at higher and lower concentrations. GH increased the number of cells in the trophoctoderm by 25%, but did not affect the inner cell mass of blastocysts. Inhibition of cell proliferation by anti-GH antiserum indicated that GH is a potent autocrine or paracrine regulator of the number of trophoctoderm cells *in vivo*. Type 1 IGF receptors (IGF1R) were localized to cytoplasmic vesicles and plasma membrane in the apical domains of uncompact and

compact eight-cell embryos, but were predominantly apparent in cytoplasmic vesicles of the trophoctoderm cells of the blastocyst, similar to GH receptors. Studies using α IR3 antiserum which blocks ligand activation of IGF1R, showed that IGF1R participate in the autocrine or paracrine regulation of the number of cells in the inner cell mass by an endogenous IGF-I–IGF1R pathway. However, α IR3 did not affect GH stimulation of the number of trophoctoderm cells. Therefore, GH does not use secondary actions via embryonic IGF-I to modify the number of blastocyst cells. This result indicates that GH and IGF-I act independently. GH may selectively regulate the number of trophoctoderm cells and thus implantation and placental growth. Embryonic GH may act in concert with IGF-I, which stimulates proliferation in the inner cell mass, to optimize blastocyst development.

Introduction

Growth hormone (GH) is an important embryonic growth regulator (Waters and Kaye, 2002). Perhaps the most marked evidence supporting this conclusion is that human Laron dwarf babies lacking GH receptor (GHR) are born more than two SD shorter than average (Laron *et al.*, 1972) and that GH-deficient mice have reduced litter size with smaller fetuses (Danilovich *et al.*, 1999). However, regulation of embryonic growth has traditionally been assigned to growth factors such as insulin and the insulin-like growth factors IGF-I and IGF-II.

Nonetheless, there is considerable evidence supporting a role for GH in embryo development. GHR mRNA has been detected in a range of extrapituitary fetal and placental tissues including the fetus and placenta in rats (Garcia-Aragon *et al.*, 1992); sheep embryos at day 51 (Klempt *et al.*, 1993), their trophoctoderm and syncytium (Lacroix *et al.*, 1999); and embryonic stem cells in mice (Ohlsson *et al.*, 1993). GHR mRNA and protein are

present in rats in multiple tissues before differentiation of the fetal pituitary (Garcia-Aragon *et al.*, 1992). GH stimulates DNA synthesis in both the fetus and placenta of rats (Botero-Ruiz *et al.*, 1997) and a variety of physiological responses demonstrate that isolated fetal tissues respond to GH (Nardo *et al.*, 2000; Phornphutkul *et al.*, 2000).

Proliferative responses to GH after birth are often mediated by IGF-I, the gene sequence of which contains a GH response activation site (Bichell *et al.*, 1992). Mice deficient in either IGF-I or type 1 IGF receptors (IGF1R) show retarded fetal growth or die soon after birth (Liu *et al.*, 1993; Baker *et al.*, 1996). In addition to GH and GHR, mouse embryos express IGF-I mRNA (Doherty *et al.*, 1994) and IGF1R mRNA (Rappolee *et al.*, 1992; Schultz *et al.*, 1992). Indeed, many of the preimplantation effects of IGF-I on embryos (Kaye *et al.*, 1992) resemble those of GH, indicating that GH may act via IGF-I.

The first embryonic cell proliferation and differentiation occurs before implantation while the embryo is moving through the reproductive tract. Over about 4 days, the mouse zygote cleaves and develops to a blastocyst consisting of a tight epithelial trophoctoderm surrounding the inner cell mass (ICM) and blastocyst cavity. GHR is expressed by mouse zygotes and persists

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throughout preimplantation development (Terada *et al.*, 1996; Pantaleon *et al.*, 1997). GH mRNA first appears in morulae and the protein is localized to outer or apical cell membranes and cytoplasmic vesicles of blastocyst trophoctoderm cells (Pantaleon *et al.*, 1997). Furthermore, exogenous GH accelerates glucose transport and protein synthesis (Pantaleon *et al.*, 1997) in an extremely sensitive and characteristic dose-dependent manner, with maximum effect at 0.1 ng ml^{-1} for bovine GH. GH supplementation *in vitro* increases the number of mouse blastocyst cells (Fukaya *et al.*, 1998) and the rate of blastocyst formation (Drakakis *et al.*, 1995; Montag *et al.*, 2000). In cattle, GHR is expressed from day 2 and GH is expressed from day 8 (Kolle *et al.*, 1998a,b, 2001; Izadyar *et al.*, 2000). GH causes an increase in glycogen and lipid metabolism in bovine blastocysts (Kolle *et al.*, 2001) and increases the number of cells in both the trophoctoderm and ICM, apparently by an inhibition of apoptosis (Kolle *et al.*, 2002). Whether this effect in cows also applies to mice, which are much more sensitive to GH and form a much smaller blastocyst, is unknown. Fertilization, cleavage rate and preimplantation outcome correlate with follicular fluid GH concentration (Mendoza *et al.*, 1999) in human embryos generated by intracytoplasmic sperm injection. These data clearly implicate GH in the modulation of preimplantation development. A point of confusion arises from the use of human GH at much higher concentrations in mouse studies (Drakakis *et al.*, 1995, 1996; Fukaya *et al.*, 1998; Montag *et al.*, 2000). These high-dose studies in mouse embryos should be repeated with non-primate GH, as primate GH also activates the mouse prolactin receptor.

The functional relationship between GH and IGF-I in preimplantation embryos has not been investigated. Although GH receptors are expressed in cleavage stage embryos and in the trophoctoderm and ICM cells of blastocysts (Pantaleon *et al.*, 1997), similar studies localizing IGF1R are lacking. Furthermore, the target tissue for GH in the mouse blastocyst is not known, nor is it known whether embryonic GH or IGF-I act via autocrine or paracrine pathways to stimulate embryonic growth. The present study was designed to answer these questions.

Materials and Methods

Embryo culture

Quackenbush mice were randomly bred (lights on from 06:00 to 18:00 h) and used for experimentation with approval of the University of Queensland Animal Ethics and Experimentation Committee. Females (10 weeks of age) were superovulated by i.p. injection of 10 iu eCG at 10:00 h followed 48 h later by 10 iu hCG (Folligon & Chorulon, Intervet, North Ryde) and paired individually with males. Mating was deter-

mined by the presence of a vaginal plug at 09:00 h the following morning. Two-cell embryos were collected 48 h after injection with hCG and washed extensively in M2 (Fulton and Whittingham, 1978), modified according to Hobbs and Kaye (1985) to remove free hormones. The embryos were then cultured for 48–52 h in groups of different density in 20 μl drops of BMOC2 (Brinster, 1965), modified according to Pemble and Kaye (1986), under liquid paraffin oil (Caltex Australia, Brisbane) at 37°C in a humidified atmosphere of 5% CO_2 , 95% air. In these conditions, two-cell embryos form blastocysts at the same rate as *in vivo*. Culture drops were supplemented as necessary with IGF-I (Gro-pep, Adelaide); recombinant bovine GH (rbGH, provided by A. F. Parlow, National Hormone and Peptide Program, NIDDKD, Harbor-UCLA Medical Centre, Torrance, CA); αIR3 , an anti-IGF1R monoclonal antibody which blocks ligand binding to IGF1R (Calbiochem-Novabiochem, Croydon); or anti-rat GH ovine antiserum (αrGH , a gift from D. Flint, Hannah Research Institute, Ayr) which binds rodent, but not bovine, GH (Flint and Gardner, 1993). Each series consisted of three or more experiments with treatment groups of at least three drops containing two or more embryos in each.

Total number of embryo cells in embryo spreads

Blastocysts (96–100 h after injection with hCG) were removed from culture drops into 1% sodium citrate for 20 min on ice, air-dried onto slides with CH_3COOH : $\text{CH}_3\text{CH}_2\text{OH}$ (1:3) and stained with giemsa (Tarkowski, 1966). Nuclei in the embryo spreads were then counted to assess the total number of cells in each embryo.

Differential staining of ICM and trophoctoderm nuclei

After culture, the nuclei of cells in the trophoctoderm and ICM of blastocysts (96–100 h after injection with hCG) were counted after differential nuclear staining (Handyside and Hunter, 1984). The zonae pellucidae were removed in acidic Tyrode's solution (pH 2.5) before incubation in 10% anti-mouse serum, prepared by immunization of a rabbit with mouse spleen cells according to Harvey and Kaye (1990), in M2 for 20 min at 37°C (Solter and Knowles, 1975). Embryos were then washed with M2 and incubated for 5 min at 37°C with 10 mg propidium iodide l^{-1} (Sigma-Aldrich, Castle Hill) and 10% dilution of guinea-pig serum (Institute of Medical and Veterinary Science, Adelaide) as a source of complement. The embryos were washed briefly in 10 mmol phosphate buffer l^{-1} (pH 7.2) containing 137 mmol NaCl l^{-1} and 2.7 mmol KCl l^{-1} (PBS) before being transferred to absolute ethanol with 25 mg bis-benzimide l^{-1} (Hoechst 33258; Sigma-Aldrich), and were then left overnight at 4°C. The morning after, the embryos were washed with ethanol before mounting in a hanging drop of glycerol and were visualized under a

Zeiss Photomicroscope 2 fitted with a HBO 50W high-pressure mercury lamp. The trophoctoderm nuclei were stained red using an excitation filter set comprising band pass interference exciter filter 546/12, barrier filter LP 590 and chromatic beam splitter FT 580 (Zeiss). The nuclei of both cell populations were visible with blue-violet excitation (filter set comprising band pass interference exciter filter G 365, barrier filter LP 420 and chromatic beam splitter FT 395; Zeiss); the trophoctoderm nuclei were pink and the ICM nuclei blue (Harvey and Kaye, 1990).

Whole mount immunostaining for IGF1R

Embryos were collected 72 and 96 h after injection with hCG, fixed in 2% paraformaldehyde in PBS for 20 min at room temperature (27°C), then washed in PBS before being placed on CellTak (Collaborative Biomedical Products, Bedford, MA) coated chambers. Fixed embryos were permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) in PBS for 15 min at room temperature, neutralized with 0.05 mol NH₄Cl l⁻¹ in PBS for 10 min, washed, then incubated in PBS containing 10% normal goat serum (NGS, obtained from goats bred at the laboratory), 1% Tween 20 (ICN Biomedicals Australasia, Pty Ltd, Seven Hills) and 4 g bovine serum albumin l⁻¹ (Trace Scientific Ltd, Noble Park, Victoria) for 60 min (NGS-Tween-BSA-PBS). Blocked embryos were incubated at 4°C overnight with 0.13% rabbit polyclonal IgG raised against the extracellular amino terminal of the α chain of the IGF1R that does not crossreact with the β chain or the IGF1R (product number sc-712; Santa Cruz Biotechnology, Santa Cruz, CA), diluted in 5% NGS-Tween-BSA-PBS. After incubation in primary antibody, embryos were washed in Tween-BSA-PBS and exposed for 60 min at room temperature to Texas-red conjugated secondary antibody (Calbiochem-Novabiochem, Sydney) diluted in PBS. Finally, after washing with PBS, embryos were mounted in CitiFluor anti-fade solution (CityFluor Ltd, London) and viewed under a Bio-Rad MRC600 confocal scanning laser microscope mounted on a Zeiss Axioskop equipped with a Zeiss Plan-Apochromat $\times 40$ oil-immersion objective.

Statistical analysis

Data were analysed by factorial ANOVA for treatment, experiment and interaction effects. Differences between treatment means were tested for significance using Fischer's least squares significant difference *post hoc* test with the Statgraphics statistical package (Manugistics Inc, Rockville, MD). Means are presented with SEM. Categorical data are presented as percentage blastocyst formed and analysed using chi-squared test in Prism 3 for Macintosh (GraphPad Software Inc., San Diego, CA).

Results

Localization of IGF1R in preimplantation embryos

The high resolution of the laser scanning confocal microscope reveals the distribution of IGF1R in embryos in whole mount (Fig. 1). Although occasional four-cell embryos (not shown) stained for IGF1R, IGF1R were first consistently detected in eight-cell embryos in the outer facing membrane or cortical cytoplasmic vesicles, just beneath the plasma membrane (Fig. 1a). This outer or apical localization persisted through compaction (Fig. 1a,b). A day later, IGF1R in blastocysts were mostly confined to cytoplasmic vesicles in trophoctoderm cells. There was weak vesicular staining in the ICM, and some staining of plasma membranes adjacent to trophoctoderm cells (Fig. 1c).

Effects of GH on blastocyst formation and growth: concentration response

GH markedly increased blastocyst formation. By 96–100 h after injection with hCG, in conditions of low density (five embryos per 20 μ l drop), 59% of two-cell embryos formed blastocysts in medium containing 1 pg rbGH ml⁻¹, almost twice as many as in the absence of GH (34%, $P < 0.01$, $\chi^2 = 8.665$, $n = 65$, 73, three experiments). The use of rbGH, which unlike primate GH does not bind to the mouse prolactin receptor, eliminated a potentially confounding effect from these studies. Accompanying this increase in blastocyst formation was a 27% increase ($P < 0.05$) in the total number of cells with 1 pg rbGH ml⁻¹ in a bell-shaped response curve, such that there was no significant response below 0.1 pg ml⁻¹ or above 10 pg ml⁻¹ (Fig. 2).

Autocrine or paracrine actions of GH

Embryos developing at a density of five embryos per 20 μ l droplet exhibited retarded growth compared with those developing in denser cultures (data not shown), indicating that endogenous autocrine or paracrine systems were diluted out when embryos were cultured in these conditions of low density. Embryos were incubated at high density (20 embryos per 20 μ l drop) to maximize autocrine or paracrine effects and 0.1% α rhGH IgG or non-immune sheep IgG was added (Fig. 3) to test whether GH was part of such an autoregulatory system. Compared with both medium alone and supplementation with non-immune sheep IgG, the blastocysts developing in the presence of α rhGH contained 13–17% fewer cells ($P < 0.05$).

Differential effects of rbGH on blastocyst cell populations

As all cells of the embryo express GH receptors, the tissue lineage, that is, ICM or trophoctoderm that

Fig. 1. Localization of type 1 insulin-like growth factor receptors in preimplantation mouse embryos. (a) Eight-cell embryo collected 72 h after injection with hCG; (b) early morula collected 72 h after injection with hCG; (c) blastocyst collected 96 h after injection with hCG; (d) normal rabbit IgG used as primary antibody for staining blastocysts 96 h after injection with hCG. Bright green colouring indicates most intense staining. Scale bar represents 25 μm .

responded to GH with increased proliferation, was determined. Blastocysts developing from the two-cell stage with 1 pg rbGH ml^{-1} were differentially stained and cell populations were counted. The trophectoderm of GH-treated blastocysts contained five cells more than the untreated blastocysts (25%, $P < 0.01$, Fig. 4). Although there was an increase of approximately one cell (about 8%) in the ICM, this was not significant (Fig. 4, $P > 0.05$). Thus, rbGH selectively stimulated trophectoderm cell population growth with no effect on ICM growth.

Does αIR3 block embryonic IGF-I: an autocrine or paracrine IGF-I circuit?

In addition to neutralizing the activity of human IGF1R, αIR3 also effectively prevents signalling mediated by IGF1R in preimplantation mouse embryos (Chi *et al.*, 2000). It was necessary to show that αIR3 blocks the effects of IGF-I on ICM growth, to resolve effects arising directly through activation of GHR from those arising subsequent to GH-induced IGF-I activation of IGF1R. A preliminary experiment indicated that

Fig. 2. Concentration response of number of mouse cells per blastocyst to recombinant bovine growth hormone (rbGH). Two-cell embryos (48 h after injection with hCG) were collected and cultured for 96–100 h after injection with hCG, at five embryos per 20 μ l in BMOC2, with different concentrations of rbGH and number of cells per blastocyst. * $P < 0.05$ by Fischer's test after two-way ANOVA for three experiments and concentrations ($\geq$ three blastocysts per concentration per experiment).

Fig. 3. Effects of anti-growth hormone (GH) antibody on the number of mouse cells per blastocyst. Two-cell embryos (48 h after injection with hCG) were collected and cultured for 96–100 h after injection with hCG, at 20 embryos per 20 μ l drop of BMOC2 (control) or BMOC2 + non-immune sheep IgG (NISS), or BMOC2 + sheep anti- α rGH IgG (anti-GH). The number of cells per blastocyst was counted. * $P < 0.05$ by Fischer's test after two-way ANOVA for experiment and treatment (four experiments with $\geq$ five blastocysts per treatment per experiment).

Fig. 4. Effects of growth hormone (GH) on mouse blastocyst cell lineages. Two-cell embryos (48 h after injection with hCG) were cultured in groups of five per 20 μ l drop of BMOC2 with 0 ($\square$) or 1 pg recombinant bovine GH ml⁻¹ ($\blacksquare$) for 96–100 h after injection with hCG. The numbers of cells in the inner cell mass (ICM) and trophectoderm were counted. ** $P < 0.01$ by Fischer's test after two-way ANOVA for experiment (six) and treatment ($>$ four blastocysts per treatment in each).

whereas 1 μ g α IR3 ml⁻¹ reduced the number of cells compared with BMOC2 alone (11 $\pm$ 1, 8 blastocysts versus 13 $\pm$ 1, 7 blastocysts), 10 μ g α IR3 ml⁻¹ produced a significant decrease (10 $\pm$ 1, 10 blastocysts, ANOVA, $P < 0.05$).

IGF-I stimulated the number of ICM cells by about two cells (20%, $P < 0.05$) and this was completely blocked by simultaneous presence of 10 μ g α IR3 ml⁻¹ throughout the culture (Fig. 5b), confirming that mouse ICM IGF1R reflects α IR3 susceptibility characteristic of other tissues (Fig. 5). The data also show that culturing embryos in the presence of α IR3 but without IGF-I produced blastocysts with 10% ($P < 0.05$) fewer ICM cells than those developing in unsupplemented medium. IGF-I was ineffective in the presence of α IR3 (Fig. 5b). Analysis of the number of trophectoderm cell responses to IGF-I and α IR3 confirmed that these cells were refractive of IGF-I (Fig. 5c). There was no significant change in the number of trophectoderm cells due to the presence of exogenous IGF-I, nor was α IR3 effective on trophectoderm, alone or in combination with IGF-I. Thus, α IR3 neutralized embryonic response to both endogenous and exogenous IGF-I, allowing it to be used to discriminate effects of GH mediated through IGF-I from effects resulting directly from GH receptor activation.

Fig. 5. Effects of insulin-like growth factor I (IGF-I) and α IR3 anti-type 1 IGF receptor (IGF1R) antibody on the number of mouse cells per blastocyst. Two-cell embryos (48 h after injection with hCG) were collected and cultured in BMOC2 (control), BMOC2 + 1.7 nmol IGF-I l^{-1} (IGF-I), BMOC2 + α IR3 anti-IGF1R antiserum (α IR3) or BMOC2 + 1.7 nmol IGF-I l^{-1} + α IR3 (IGF-I- α IR3). (a) Total and differential cell counts in (b) inner cell mass (ICM) and (c) trophectoderm were performed at 96–100 h after injection

Does IGF-I downstream of GH stimulate trophectoderm cell proliferation?

Embryos cultured in the presence of 1 pg GH ml^{-1} contained about seven more cells than control blastocysts (25%, $P < 0.01$, Fig. 6a; see also Fig. 2). This effect of GH on total number of cells persisted in the presence of α IR3. Blocking IGF1R had no impact on the GH-stimulated blastocyst growth (Fig. 6). The hypothesis that GH induces synthesis of IGF-I and its subsequent stimulation of IGF1R, was tested by counting separately the blastocyst trophectoderm and ICM populations after stimulation with GH in the presence of α IR3. GH did not affect the ICM, whereas addition of α IR3 inhibited ICM growth by about 20% ($P < 0.01$, Fig. 6b). GH completely overcame this α IR3-induced inhibition of ICM growth (Fig. 6b). In the trophectoderm, 1 pg GH ml^{-1} promoted growth of five or six more cells (33%, $P < 0.01$), irrespective of the presence of α IR3, which was ineffective alone (Fig. 6c).

Discussion

The results of the present study reveal GH to be an endogenous growth factor for trophectoderm cell proliferation via GHR, excluding participation of IGF-I. IGF1R were distributed to the apical domains of the morulae and cytoplasm of trophectoderm cells, indicating that they were arrayed to receive ligands secreted by the maternal epithelium or by the embryo itself. Operation of an endogenous IGF-I-IGF1R pathway was also demonstrated by the studies with the IGF1R blocking antibody, α IR3.

Although IGF1R mRNA expression at the morula stage has been described in mice (Rappolee *et al.*, 1992), studies of expression and localization of the protein in mouse embryos are limited. In morulae, IGF1R were concentrated in the apical or outer facing domains of the embryo. Upon blastocyst formation, IGF1R were predominantly found in cytoplasmic vesicles in trophectoderm cells, reflecting apical location in the morulae. Supporting these findings, an earlier immunoelectron microscopic study of blastocysts found IGF1R located in the basolateral membranes and cytoplasmic vesicles of trophectoderm cells (Smith *et al.*, 1993). Furthermore, under IGF-I treatment, IGF1R were also present in the apical trophectoderm membranes (Chi *et al.*, 2000). As IGF-I exclusively stimulates the number of cells of the ICM (Harvey and Kaye, 1992), this implies that the trophectoderm receptors transcytose IGF-I across the trophectoderm to bind to ICM receptors, leading

with hCG. Means with the same letter are significantly different; $P < 0.05$ by Fischer's test after two-way ANOVA for experiment and treatment (three experiments with $\geq$ six blastocysts per treatment per experiment).

Fig. 6. Effects of growth hormone (GH) and α IR3 anti-type 1 IGF receptor (IGF1R) antiserum on the number of mouse cells per blastocyst. Two-cell embryos (48 h after injection with hCG) were collected and cultured in BMOC2 (control), BMOC2 + 1 pg recombinant bovine GH (rbGH) ml^{-1} (GH), BMOC2 + α IR3 anti-IGF1R antiserum (α IR3), or BMOC2 + 1 pg rbGH ml^{-1} + α IR3 (GH- α IR3). Cells in blastocysts were counted at 96–100 h after injection with hCG. (a) Total and differential cell counts

to an increased number of ICM cells. Movement of labelled IGF-I along this pathway has been visualized using electron microscopy (Smith *et al.*, 1993).

In the present study, GH markedly increased the proportion of embryos forming blastocysts and the number of cells in those blastocysts. In particular, GH selectively targeted growth of the trophoblast population independently of IGF-1R action, producing a tissue with 25% more cells. The growth of mouse trophoblast is extremely sensitive to GH, with maximum response in the range of 0.1–10 pg ml^{-1} , at least one order of magnitude more sensitive than in earlier reports. The present results confirm other reports that GH-induced increases in the number of blastocyst cells arise through actions mediated by the mouse GH receptor (Fukaya *et al.*, 1998). The characteristic bell-shaped dose-response curve for rbGH and the sensitivity of the embryonic receptor signalling system eliminate any possibility that GH actions were mediated by crossreaction with the mouse prolactin receptor. This was a possible confounder in earlier results which used higher doses of human GH (Drakakis *et al.*, 1995, 1996; Fukaya *et al.*, 1998) that may bind to the mouse prolactin receptor. The high sensitivity of the mouse GH receptor in the present study may have arisen from the experimental conditions, including the use of the simple medium BMOC. However, others have reported similar effects in mice as well as cattle, using more complex media including medium 199 and Ham's F10 (Drakakis *et al.*, 1995, 1996; Fukaya *et al.*, 1998; Kolle *et al.*, 2001, 2002).

An important developmental observation in the present study is that GH caused growth of the epithelial trophoblast, formed by the first round of cellular differentiation during preimplantation development. This epithelium generates ionic and osmotic gradients that draw extracellular fluid into the morula creating the blastocyst cavity. The blastocyst cavity is essential for implantation as it provides the necessary topography on which subsequent differentiation occurs. Cells developing from the trophoblast invade the maternal endometrium, providing direct access to maternal nutrients necessary for continued embryo growth and development. Thus, GH may have an important role in promoting blastocyst formation and implantation. In support of this concept, blastocyst hatching from the zona pellucida is accelerated by GH (Drakakis *et al.*, 1996; Fukaya *et al.*, 1998; Montag *et al.*, 2000) and greater numbers of blastocysts that develop *in vitro* in the presence of GH than untreated blastocysts implant after embryo transfer (Fukaya *et al.*, 1998). GH induces expression of matrix metalloproteinases

in (b) inner cell mass (ICM) and (c) trophectoderm. ** $P < 0.01$ by Fischer's test after two-way ANOVA for experiment and treatment (three experiments with $\geq$ four blastocysts per treatment per experiment).

(MMP) (Thompson *et al.*, 2000) that may be involved in implantation (Harvey *et al.*, 1995a; Whiteside *et al.*, 2001). Perhaps GH, like leukaemia inhibitory factor, is another soluble factor stimulating secretion of MMP from trophoctoderm cells (Harvey *et al.*, 1995b), facilitating hatching and implantation.

The simultaneous presence in the eight-cell morula of the four participants in a GH-IGF-I paracrine loop present the possibility that IGF-I and IGF1R act as intermediates in trophoctoderm hyperplasia promoted by GH. However, because the IGF1R blocking antibody (α IR3) and GH affect the number of cells of different blastocyst cell populations, this possibility is highly unlikely. The other reported effects of GH during preimplantation embryogenesis are also functions of the trophoctoderm; that is, stimulation of glucose uptake (Pantaleon *et al.*, 1997) and blastocyst formation (the present study; Drakakis *et al.*, 1995; Fukaya *et al.*, 1998; Montag *et al.*, 2000). GH therefore appears to be targeted specifically at modifying trophoctoderm physiology, whereas IGF-I affects both cell populations but stimulates only ICM growth (Kaye *et al.*, 1992; Kaye and Harvey, 1995; Kaye, 1997).

In human (Spanos *et al.*, 2000), rabbit (Herrler *et al.*, 1998) and mouse embryos (Chi *et al.*, 2000), IGF-I increases ICM growth by inhibiting apoptosis, which is a more common feature of the ICM than trophoctoderm (Pampfer, 2000). Therefore, it is more likely that growth in the trophoctoderm population, induced by GH, arises from proliferative actions rather than anti-apoptotic actions, although this remains to be determined. Indeed, in bovine embryos, GH stimulates growth of both the ICM and trophoctoderm through inhibition of apoptosis (Kolle *et al.*, 2002). Blocking IGF1R of otherwise unsupplemented embryos causes the development of blastocysts with a smaller ICM. Thus, an embryonic IGF-I-IGF1R circuit inhibits apoptosis in preimplantation embryos. It has been suggested that IGF-I expression (presumably acting in this circuit) correlates with embryo viability after culture and transfer (Kowalik *et al.*, 1999). It is clear that activation of such an endogenous embryonic IGF-I loop is not the means by which GH stimulates trophoctoderm hyperplasia.

The present study has shown that, although ineffective on the ICM of embryos cultured with an intact IGF1R, GH could compensate for the α IR3 blockade on ICM growth. This might reflect an anti-apoptotic effect of GH on ICM (Kolle *et al.*, 2002). GH is anti-apoptotic in other cell lines (Costoya *et al.*, 1999; Baixeras *et al.*, 2001). Perhaps GH or an intermediate factor crosses the trophoctoderm to maintain cell proliferation by inhibiting apoptosis in the ICM.

In conclusion, GH has been shown to be an important preimplantation embryonic growth factor promoting proliferation of the trophoctoderm with implications for subsequent implantation of the blastocyst. This activity is independent of IGF1R. In contrast, IGF-I promotes

ICM growth through an autocrine or paracrine pathway. The operation of this IGF-I pathway is reflected in the localization of embryonic IGF1R to the outer facing domains of cleaving and compacting embryos and to the trophoctoderm. Demonstration of these endogenous pathways of GH and IGF-I actions has important implications in the understanding of the physiology of early pregnancy and indicates potential improvements in media for *in vitro* culture of embryos.

Note added in proof: Moreira *et al.* (2002) used anti-IGF-I rather than anti-IGF1R to show that GH acts independently of IGF-I to increase the number of cells in bovine blastocysts.

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