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IGF-I and insulin activate mitogen-activated protein kinase via the type 1 IGF receptor in mouse embryonic stem cells

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Abstract

Although IGF-I and insulin are important modulators of preimplantation embryonic physiology, the signalling pathways activated during development remain to be elucidated. As a model of preimplantation embryos, pluripotent mouse embryonic stem cells were used to investigate which receptor mediated actions of physiological concentrations of IGF-I and insulin on growth measured by protein synthesis. Exposure of mouse embryonic stem (ES) cells to 1.7 pM IGF-I or 1.7 nM insulin for 4 h caused ~25% increase in protein synthesis when compared with cells cultured in basal medium containing BSA. Dose–response studies showed 100-fold higher potency of IGF-I that pointed to the type 1 IGF receptor as the mediating receptor for both ligands. This was confirmed using an anti-type 1 IGF receptor-blocking antibody (αIR3). Both 1.7 pM IGF-I and 1.7 nM insulin increased phosphorylation of the type 1 IGF receptor and this increase was blocked by αIR3, but the insulin receptor was not phosphorylated. Finally, binding of either agonist led to downstream phosphorylation of ERK1/2 mitogen-activated protein kinase (MAPK) also via IGF-1R as this was blocked by αIR3. Together, these results suggest that IGF-I and insulin modulate ES cell physiology through binding to the type 1 IGF receptor and subsequent activation of MAPK pathway.

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Introduction

All members of the insulin-like family of growth factors (IGF) have been shown to modulate preimplantation physiology in mouse embryos (Kaye 1997). For example, in the blastocyst, insulin and IGF-I caused an increase in inner cell mass (ICM) proliferation and protein synthetic rate (Harvey & Kaye 1988, 1990, 1991*a*, 1992*a*, 1992*b*)). Both the insulin (IR) and type 1 IGF receptors (IGF-1R) are expressed by mouse preimplantation embryos including the blastocyst ICM (Harvey & Kaye 1991*b*, Markham & Kaye 2003). During preimplantation development, activation of either IGF-1R or IR is associated with metabolic and mitogenic responses *in vitro* (Kaye *et al.* 1992).

Insulin and IGF-I are closely related cytokines, whose biological effects are mediated by binding to their respective cell surface type 1 tyrosine kinase receptors. The receptors are heteromeric transmembrane receptors, consisting of 2α and 2β subunits linked by disulphide bonds (Rechler & Nissley 1985). Binding of ligand to the α-subunit leads to autophosphorylation of tyrosine residues within the β-subunits (Seely *et al.* 1995) and coupled activation of a complex network of intracellular signalling pathways leading to divergent biological cellular responses (Avruch 1998). Traditionally, metabolic outcomes are attributed to signalling via the phosphatidylinositol 3-kinase pathway and the mitogenic response is attributed to the ERK mitogen-activated protein kinase (MAPK) pathway (Taniguchi *et al.* 2006). Whilst there is receptor crosstalk, the prevailing view is that the IR tends to regulate metabolic activities related to glucose and energy homeostasis, whilst the IGF-1R is mainly involved in regulation of cell proliferation, anti-apoptosis, differentiation and cell motility (Rother & Accili 2000).

During development, the physiological roles of IGF-I and insulin are not clear (Kaye 1997) and even in embryonic stem (ES) cells little is known of the linkages between potential intracellular signalling pathways and cellular growth responses during development. In rabbit blastocysts, insulin activated the MAPK/ERK pathway (Navarrete Santos *et al.* 2004), suggesting that its actions were directed at promotion of proliferation and growth rather than regulation of glucose metabolism in these embryos. Whilst this has been suggested to be the case in mouse, there is little direct evidence to identify the mediating receptor or the signalling pathway activated.

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The availability of large numbers of pluripotent mouse ES (mES) cells (Nagy *et al.* 1993, Wood *et al.* 1993) permits molecular investigations into signalling and physiological function of these receptors, not readily available in mouse embryos. We therefore investigated the signalling pathway in mR1ES cells from brief exposure to physiological concentrations of IGF-I or insulin as a model for the growth response of embryonic cells.

Materials and Methods

Antibodies

The following antibodies were used: rabbit polyclonal anti-IGF-1R α-subunit (N-20: sc-712), anti-IGF-1R β-subunit (catalogue no. C-20: sc-713), anti-IR α-subunit (H-78: sc-7953), anti-IR β-subunit (catalogue no. C19: sc-711) and anti-ERK1/2, a non-active form of MAPK (catalogue no. C16: sc-93) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); monoclonal anti-phospho-ERK1/2 antibody (phospho-p42/p44 MAPK Thr202/Tyr204, catalogue no. 9101S) from Cell Signalling Technologies Co. (Boston, MA, USA); monoclonal anti-mouse phosphotyrosine antibody (4G10, catalogue no. 16–105) and normal rabbit immunoglobulin G (IgG; catalogue no. 12–370) from Upstate Biotech (Charlottesville, VA, USA); horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (catalogue no. NA934) and HRP-conjugated sheep anti-mouse IgG (catalogue no. NA931) from Amersham Biosciences; αIR3 (catalogue no. GR11L, anti-IGF-1R-blocking antibody) from Oncogene Research Products (San Diego, CA, USA) and isotype-matched mouse IgG1 as a negative control antibody for αIR3 (catalogue no. MOPC-21) from Sigma Chemical Co.

Cell culture

Mouse R1ES ES cells were chosen for their demonstrated pluripotency. Specifically, R1ES cell pluripotency has been tested by tetraploid embryo mES aggregates for entire ES-derived development (Nagy *et al.* 1993), by diploid embryo ES aggregates and blastocyst injection for germline transmission in chimeras (Wood *et al.* 1993; Gautam *et al.* 1996; Patton *et al.* 2001). They are male ES cells in a line established from (129/Sv×129/Sv-CP) F1 mouse blastocysts and were a gift from Dr Nagy, University Samuel Lunfeld Research Institute, Canada (Nagy *et al.* 1993).

These mES cells were obtained from Dr Nagy at passage 10. After expansion cells at passage 14 were cultured for three passages to 70% confluency in the absence of irradiated mouse embryonic fibroblasts (*i*MEF) in ES media (Dulbecco's modified Eagle's medium (DMEM; Invitrogen), containing 10^6 units recombinant human leukaemia inhibitory factor per litre (LIF, ESGRO Silenus, Chemicon International, Boronia, Australia), 5% (v/v) heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT, USA), 2 mM L-glutamine, 0.1 mM non-essential amino acids (Invitrogen), 100 mg streptomycin/penicillin per litre (Invitrogen) and 0.24 mM β-mercaptoethanol (Sigma Chemical Co). Prior to serum deprivation, 20 000 cells per well were cultured in the same medium but with 0.5% FCS for 12–24 h. R1ES cells showed no evidence of differentiation indicated by the following morphological criteria: the presence of ectodermal cells, loss of cell–cell contact, flattening of cells with numerous lamellipodia and filopodial extensions around their circumference (Robertson 1987, Hogan *et al.* 1994).

Preparation of cellular protein extracts

R1ES cells were cultured as described above. Following serum deprivation for 12 h, the cells were incubated for 10 min at 37 °C in an atmosphere of 7.5% CO_2 with 1.7 pM human IGF-I (Gropep, Adelaide, Australia) or 1.7 nM human insulin (Humulin R, Eli Lilly) and as a control 0.5–1.0 g BSA per litre (Fraction V, Sigma Chemical Co). After incubation, cells were washed twice with ice-cold PBS (pH 7.4) with 2 mM Na_3VO_4 and scraped off with 0.2 ml lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1% (v/v) Triton X-100 and one complete protease inhibitor tablet/50 ml of lysis buffer (catalogue. no. 1697498, Roche Diagnostics). The scraped cells were homogenised, allowed to stand on ice for 30 min and clarified by centrifugation at 15 000 ***g*** for 10 min at 4 °C. The protein concentrations of the resulting supernatants were determined by the Pierce BCA protein assay according to the manufacturer's instructions (Pierce BCA Protein Assay Kit; Pierce, Milwaukee, WI, USA).

Immunoprecipitation

Cell lysates containing 0.5 mg protein were incubated with 10 μl of either anti-IGF-1Rβ or anti-IRβ at 200 mg/l for 1.5 h with gentle shaking at 4 °C. Then 40 μl BSA-coated protein A sepharose beads were added (Amersham Biosciences) and incubation continued for another 1.5 h with gentle shaking at 4 °C. The beads were precipitated, washed thrice with cold lysis buffer at 4 °C and collected. The bound proteins were boiled in 2% (w/v) SDS sample buffer for 5 min and loaded onto 7.5% (w/v) SDS bisacrylamide gel for electrophoresis and immunoblotting analysis, using anti-mouse monoclonal phosphotyrosine antibody (4G10) at 1:1000 dilution.

Immunoblotting

Equal amounts of total protein (64 μg) for immunoblotting analysis were resolved by SDS-PAGE on 7.5%

(w/v) bisacrylamide gels, transferred onto nitrocellulose membranes by semi-dry transfer for 2 h at 32 mA, according to the manufacturer's instructions (Semi-Phor; Hoefer, San Francisco, CA, USA). The membranes were incubated for 1 h at room temperature in 0.05% (v/v) Tween 20 with 3% (w/v) BSA in Tris-buffered saline (TBS; pH 7.4) before a 12-h incubation at 4 °C with one of the following rabbit affinity-purified primary antibodies diluted in TBS containing 0.05% (v/v) Tween 20 and 1% (w/v) BSA: anti-IGF-1Rβ, anti-IGF-1Rα, anti-IRα and anti-IRβ, all at 1:100 dilution or anti-phospho-ERK1/2, which was used at 1:1000. For antibody specificity control, normal rabbit IgG was used at 0.2 mg/l. After washing for 30 min in three changes of TBS, 0.1% (v/v) Tween 20, membranes were incubated for 1 h with HRP-conjugated donkey anti-rabbit IgG or HRP-sheep anti-mouse IgG at 1:10 000, washed four times in TBS, 0.1% (v/v) Tween 20 and immunoreactive bands were visualised using ECL according to the manufacturer's instructions (Pierce Supersignal West Pico Chemiluminescent Kit; Pierce). After autoradiography, the intensity of each immunoblot band was quantified using Photoshop (Version 9.0; Adobe Systems). After detection of phosphorylated proteins, to confirm the identity of the bands and assess protein loading, membranes were stripped of bound antibodies and reprobed with the receptor or kinase-specific antibodies. Stripping of bound antibody was by incubation at 60 °C with gentle agitation in stripping buffer (56.25 mM Tris–HCl, pH 6.7; 2% (w/v) SDS, 100 mM 2β-mercaptoethanol) for 30 min. After washing, membranes were blocked with 5% skim milk in TBS for 1–2 h at room temperature and incubated with specific primary antibody diluted 1:1000 in TBS containing 0.05% (v/v) Tween 20 and (1% w/v) BSA overnight at 4 °C. Immunodetection by chemiluminescence was as above.

mES cell protein synthesis bioassay

R1ES cells were cultured as described above, and after three passages, placed into 0.1% (w/v) gelatinised wells of 96-well tissue culture plates at a density of 20 000 cells per well. They were cultured in serum-free ES medium for 12 h prior to treatment for 4 h with IGF-I or insulin at the concentrations, IGF-I: 0.17, 1.7, 17 and 170 pM; insulin: 0.17 pM, 1.7 pM, 17 pM, 170 pM, 1.7 nM and 17 nM, before the media were replaced with media containing 0.1 TBq [4,5-^{3}H]leucine (7.4 TBq/mmole, Amersham Biosciences), DMEM containing 0.24 mM 2β-mercaptoethanol, 0.1 mM non-essential amino acids, 100 mg penicillin–streptomycin, 2 mM L-glutamine and 1 g BSA l^{-1}, and incubation continued for 2 h. Medium was removed and cells resuspended in 200 μl of 2.5 μM trypsin–EDTA, 1 g BSA l^{-1}was added and cells transferred to tubes containing 0.5 ml cold 20% (w/v) trichloroacetic acid (TCA) on ice. Tubes were mixed immediately and allowed to stand for 30 min on ice before collecting the protein precipitates on glass fibre discs. After washing with ice-cold 10% TCA, the discs were transferred to scintillation vials, 1.0 ml scintillation cocktail (OptiPhase 'HiSafe'; Wallac and Berthold, Milton Keynes, UK) added and radioactivity was analysed.

To assess the effect of IGF-I-blocking antibody on the stimulation of R1ES protein synthesis, cells were incubated with and without 0.1 mg αIR3 l^{-1} (IGF-1R-blocking antibody) for 1 h, followed by a further 4-h incubation either with or without 1.7 pM IGF-I or 1.7 nM insulin at 37 °C in 7.5% CO_2. As a negative control for αIR3, an equivalent concentration of isotype-matched mouse IgG1 was used.

Statistical analysis

Statgraphics (Version 3.0. Manugistics Inc., Scottsdale, AZ, USA) was used for factorial ANOVA and multiple means range post-tests using Fisher's protected procedure and linear regression analysis for all the bioassays. Untransformed least square means with standard error mean (S.E.M.) are presented in all graphs and text.

Results

Expression of IGF-1R and IR by mES cells

Previous studies indicated that IGF-1R and IR mRNA were expressed in mES cells (Shi *et al.* 1995). We therefore sought to confirm the expression of the proteins in R1ES cells under our specific growth conditions. Immunoblotting analyses on R1ES whole-cell lysates, using specific antibodies that recognise the individual α- and β-subunits of the IGF-1R and IR, were used. Two isoforms of IGF-1R β-subunit at mobility equivalent to ~97 and 105 kDa were detected in R1ES cells, whereas only the ~105 kDa isoform was detected in embryonic day 18 (E18) skeletal muscle used as the control (Fig. 1A). The IGF-1R α-subunit immunoblots revealed prominent bands at ~130 kDa for R1ES cells and ~132 kDa in E18 muscle (Fig. 1B). We found apparently less expression of IR than IGF-1R using whole-cell extracts (Fig. 1C and D). Blotting with anti-IR β-subunit revealed a weak band at ~97 kDa in R1ES cells that was more intense in E18 muscle (Fig. 1C). However, when probed with anti-IR α-subunit, a similar pattern to IGF-1R emerged, weak bands at ~130 and 132 kDa for R1ES cells and stronger band at 130 kDa in E18 muscle with mobility (Fig. 1D). There were no detectable bands when either R1ES cells or E18 skeletal muscle samples were probed with normal rabbit IgG (Fig. 1E). These general profiles were also present for both subunits of both IGF-1R and IR in later experiments examining autophosphorylation (Fig. 4).

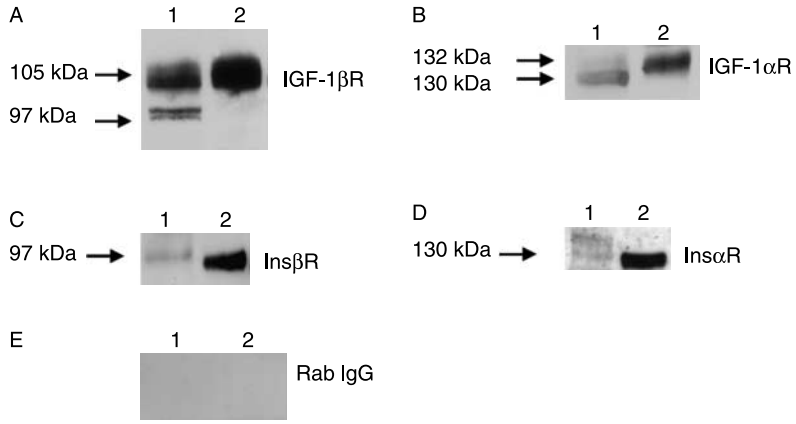

Figure 1 Expression of IGF-1R and IR by mES cells. Immunoblots of R1ES cells (lanes 1) and embryonic day 18 muscle (E18, lanes 2) were probed for the individual subunits of IGF-1R (A and B) and IR (C and D). (A) Two main forms of the IGF-1R β-subunit (IGF-1βR) in R1ES cells (105 and ~97 kDa, lane 1), whereas in lane 2, only 105 kDa form in E18 muscle. (B) The IGF-1R α-subunit (IGF-1αR) in R1ES cells (130 kDa, lane 1) and E18 muscle (132 kDa, lane 2). (C) The 97 kDa IR β-subunit (InsβR) in R1ES cells (weak immunoreactive band, lane 1) and E18 muscle (intense immunoreactive band, lane 2). (D) 130 and 132 kDa forms of IR α-subunit (InsαR) in R1ES cells and of 130 kDa form in E18 muscle (lanes 1 and 2 respectively). (E) No immunoreactive band in R1ES cells (lane 1) or E18 muscle (lane 2) probed with rabbit IgG (Rab IgG). Note: equal amounts of total protein (64 μg) from all samples were loaded.

IGF-I and insulin stimulate mES cell protein synthesis

The first step in developing mES cells as a model system to investigate signalling in embryonic cells was achieved by conducting experiments to show that a common metabolic parameter of growth, protein synthesis, responded to a short exposure to either IGF-I or insulin.

In these assays, both IGF-I and insulin caused a dose-dependent increase in R1-ES cell protein synthesis (Fig. 2). The EC_{50}, ~1.0 pM, shows that R1ES cells were exquisitely sensitive to IGF-I, which caused a 27% stimulation over a tenfold IGF-I concentration range (10^{-1}–10^{0} pM; Fig. 2A, $P<0.05$, $n=6$). Insulin produced a similar stimulation of 19%, but with only ~1% the potency of IGF-I (EC_{50}, ~130 pM) over 1000-fold concentration range (10^{0}–10^{3} pM; compare Fig. 2A with Fig. 2B, $P<0.05$, $n=7$). As another control, 5% FCS plus LIF produced 30% stimulation in all experiments (data not shown).

IGF-I and insulin act via binding to IGF-1R

The lower potency of insulin (Fig. 2) suggested that it might be activating the IGF-1R rather than the IR. To test this, we used a monoclonal IGF-1R-blocking antibody (αIR3), which binds specifically to the extracellular α-subunit of the IGF-1R and has been previously shown to block IGF-1R activation by either IGF-I or insulin (Kull *et al.* 1983, Rohlik *et al.* 1987, Chi *et al.* 2000, Markham & Kaye 2003).

Neither IGF-I nor insulin was effective on αIR3-pretreated R1ES cells (Fig. 3A and B). A specificity control using an isotype-matched mouse IgG1 was also without effect, irrespective of the presence of agonists confirming the specificity of αIR3 on R1ES cells (data not shown).

IGF-I and insulin activate the IGF-1R tyrosine kinase

To further support our observations that IGF-I and insulin were activating the IGF-1R, the phosphorylation of IGF-1R β-subunit following agonist binding was assessed. The results show a low level of activation without agonist, but increased phosphorylation of the IGF-1R with 1.7 pM IGF-I or 1.7 nM insulin (Fig. 4A). Furthermore, this phosphorylation was blocked by co-incubation with αIR3 (Fig. 4A). The identity of the phosphorylated bands at ~105 and 97 kDa as isoforms of IGF-1R β-subunit was confirmed by stripping the blot and reprobing with antibodies directed against the IGF-1R β-subunit (Fig. 4A). Densitometric scans of three replicate blots demonstrated a similar 50% increase in IGF-1R β-subunit phosphorylation by IGF-I or insulin (Fig. 4A, $P<0.05$, $n=3$, ANOVA) when compared with the basal conditions (0.5 g BSA l^{-1}). By contrast, αIR3 reduced phosphorylation by about 70% (Fig. 4A. $P<0.05$, $n=3$, ANOVA). These findings suggest that all three treatments activate the IGF-1R and not the IR. Analysis of IR phosphorylation confirmed this. While the IR could be detected, there was no evidence of IR phosphorylation and αIR3 had no effect (Fig. 4B).

IGF-1R activation leads to ERK1/2 MAPK phosphorylation

The intracellular signalling cascade from the phosphorylated IGF-1R via the extracellular signal-regulated MAP kinase (ERK1/2 MAPK) pathway was examined. Again whilst there was a low level of phosphorylation in the base medium (0.5 g BSA l^{-1}), both 1.7 pM IGF-I and 1.7 nM insulin caused 60–100% further phosphorylation of ERK1/2 (Fig. 5A), and this was effectively blocked by αIR3 (Fig. 5A). The identity of the phosphorylated bands

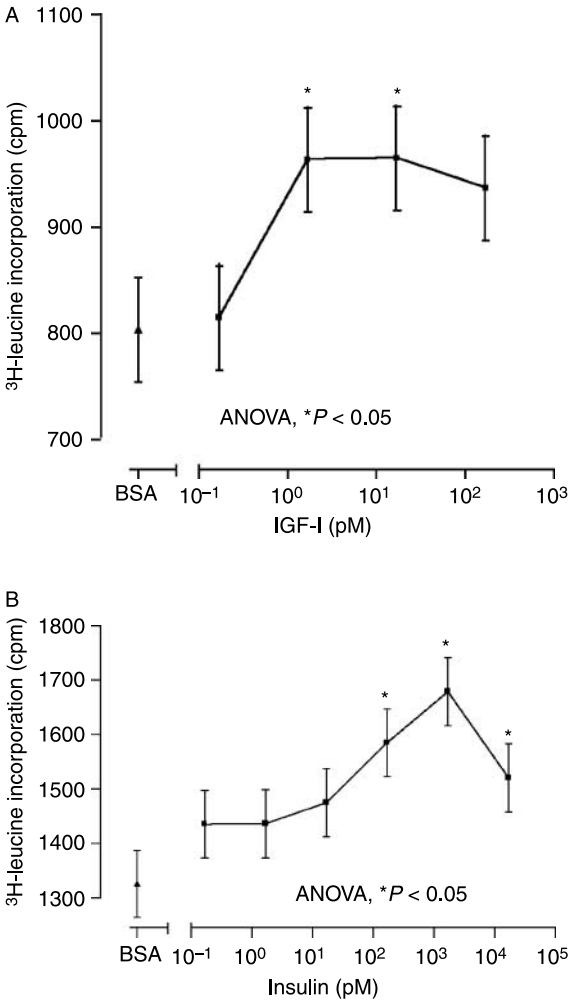

Figure 2 Dose–response curves for short-term IGF-I and insulin stimulation of protein synthesis in mES cells. Overnight serum-starved R1ES cells incubated for 4 h with (A) IGF-I or (B) insulin and 1 g BSA l^{-1} (BSA) before assay of ^{3}H-leucine incorporation over 2 h. Each point represents mean ± S.E.M. of six experiments with four replicates at each concentration of IGF-I (ANOVA *$P<0.05$, EC_{50} ≈ 1 pM) and seven experiments with four replicates at each concentration of insulin (ANOVA *$P<0.05$, EC_{50} ≈ 130 pM).

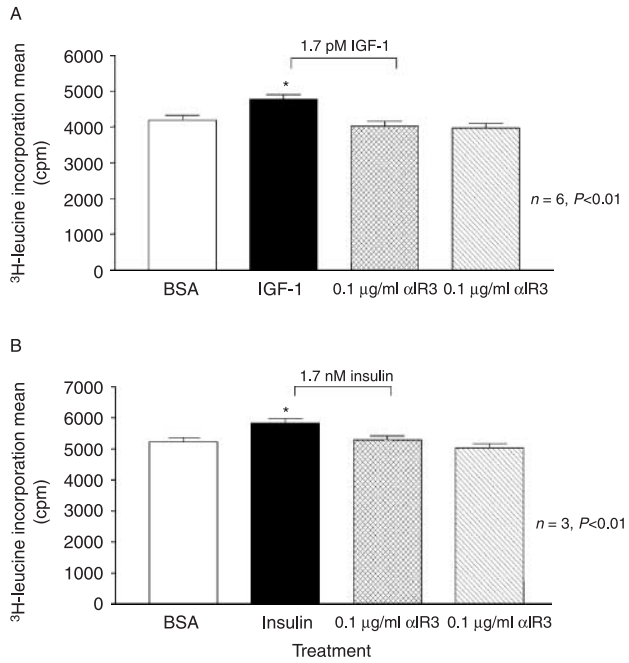

Figure 3 In mES cells, both IGF-I and insulin stimulate protein synthesis by signalling through IGF-1R. [^{3}H]leucine incorporation over 2 h by ES cells treated for 4 h with 1.7 pM IGF-I or 1.7 nM insulin in the presence of 1g BSA l^{-1} (BSA) after an incubation of 1 h with 0.1 mg αIR3 l^{-1} (IGF-1R-blocking antibody). (A) R1ES cells incubated with IGF-I and αIR3 show reduction in protein synthesis when compared with ES cells incubated with IGF-I alone (black and cross-hatched bars; *$P<0.01$, ANOVA). This level of reduction was to the same level as control media (serum-free media, 1 g BSA l^{-1}). (B) Reduced protein synthesis when ES cells were incubated with αIR3 and insulin compared with insulin alone (cross-hatched and black bars; $P<0.01$, ANOVA). Again this level of reduction was to control media levels (open and striped bars). There were no toxic effects using αIR3 on control levels of ES protein synthesis (open and striped bars in A and B). Each bar represents the mean ± S.E.M. of (A) six and (B) three experiments each with four replicates per treatment.

at 44 and 42 kDa as active ERK1/2 was confirmed by stripping the blot and reprobing with antibodies directed against the ERK1/2 (Fig. 5A). Scans of three replicate blots demonstrated that taking into account the level of unphosphorylated ERK1/2, αIR3 reduced phosphorylation of ERK1/2 initiated in all three treatments (Fig. 5B, $P<0.05$, $n=3$, ANOVA).

Discussion

The aim of this study was to examine the signalling pathway in ES cells that was activated by IGF-I or insulin leading to changes in mES cell metabolism relating to growth as reflected by changes in protein synthesis. The results confirmed that IGF-1R and IR, which are expressed in ICM cells (Harvey & Kaye 1991*b*, Markham & Kaye 2003), continue to be expressed by mES cells under these growth conditions. However, both ligands stimulate growth measured by protein synthesis via binding to the IGF-1R, not the IR, leading to increased autophosphorylation of the IGF-1R β-subunit and the MAPK pathway. This concurs with recent results from rabbit blastocysts (Navarrete Santos *et al.* 2004), which did not support a role for insulin in stimulating blastocyst glucose transport.

IGF-1R and insulin receptors are expressed by mES cells

Specific antibodies revealed that all subunits of both receptors were expressed in R1ES cells. Previous studies have shown IGF-1R and IR mRNA transcripts to be present in ES cells (Shi *et al.* 1995). We observed two forms of each of the IGF-1R subunits and of the IR α-subunit, but only one band for IR β-subunit.

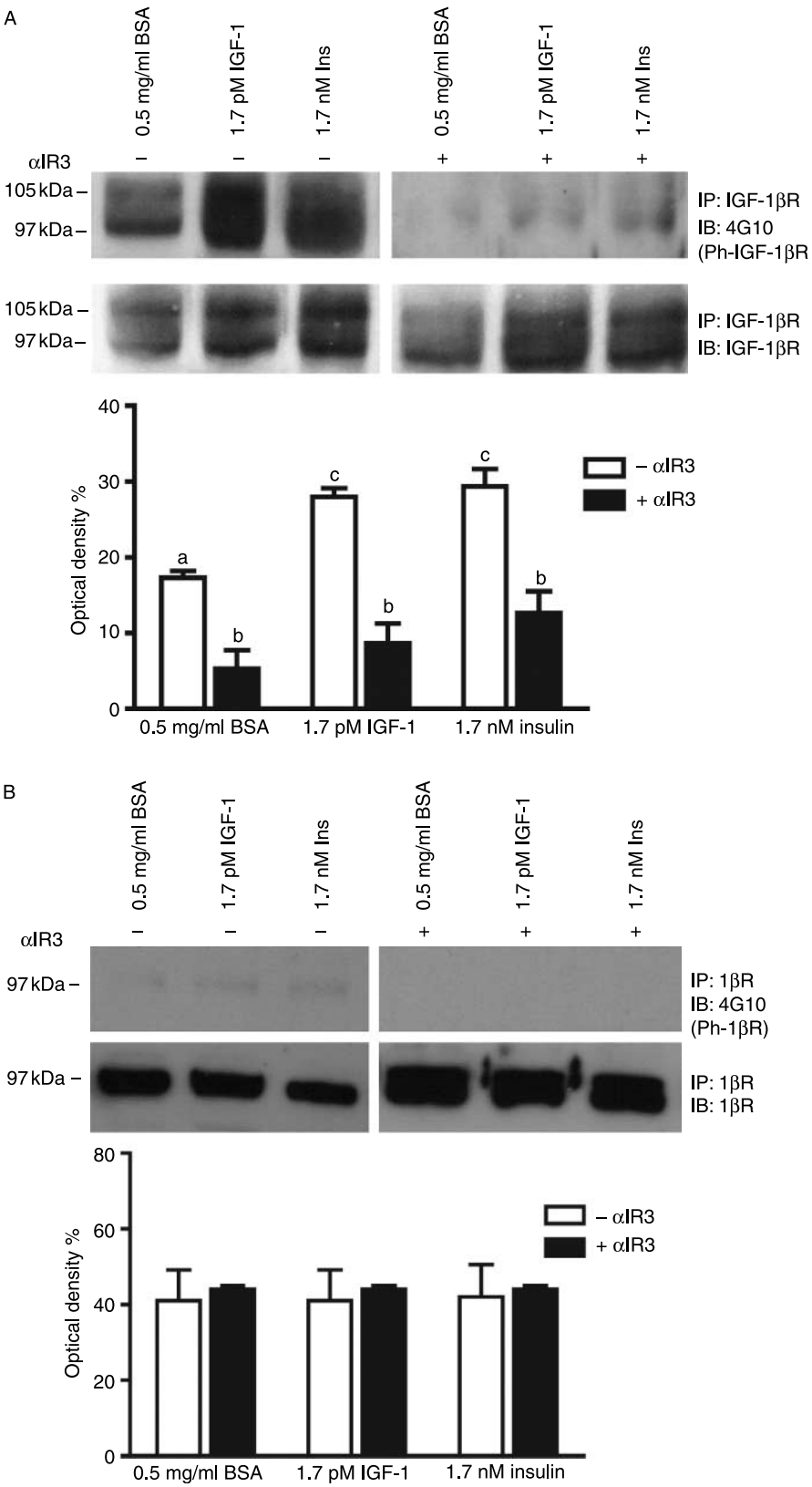

Figure 4 Autophosphorylation of IGF-1R but not IR in mES cells, by either IGF-I or insulin. (A) IGF-1R β-subunit (IGF-1βR) autophosphorylated in R1ES cells with 1.7 pM IGF-I, 1.7 nM insulin (Ins) or control basal medium containing only 0.5 g BSA l^{-1}. After stimulation, cell lysates were immunoprecipitated with anti-IGF-1R β-subunit followed by immunoblotting with an anti-phosphotyrosine antibody (4G10) to detect phosphorylated protein (left upper '−' blot tracks). The autophosphorylation was effectively blocked by the presence of αIR3 (right upper '+' blot tracks). Quantitative scans show the ratio of the optical density of the phosphorylated band as a percentage of the band stripped and blotted with the relevant receptor antibody, mean (±S.E.M., $n=3$). Note similar increases in IGF-1R phosphorylation initiated by IGF-I and insulin when compared with BSA, in the absence of αIR3 (open bars, $P<0.05$, $n=3$, ANOVA, indicated by the superscripts 'a' and 'c'). By contrast, αIR3 reduced phosphorylation of IGF-1R β-subunit caused by IGF-I, insulin and BSA (black bars, $P<0.05$, $n=3$, ANOVA, indicated by 'a' and 'c' versus 'b' superscripts). (B) R1ES cell IR β-subunit was not autophosphorylated in response to 1.7 pM IGF-I or 1.7 nM insulin (Ins). After simulation, cell lysates were immunoprecipitated with anti-IR β-subunit followed by immunoblotting with 4G10 to detect phosphorylated IR in either the absence ('−') or presence ('+') of αIR3 (left and right upper blot tracks). Quantitative scans show the ratio of the optical density of the phosphorylated band as a percentage of the band stripped and blotted with the relevant receptor antibody, mean (±S.E.M., $n=3$). Note no variation irrespective of the absence (open bars) or presence (black bars) of αIR3 ($P>0.05$, $n=3$, ANOVA). The identity of the phosphorylated bands as IGF-1R or IR β-subunit was demonstrated by stripping and reprobing with either anti-IGF-1R β-subunit (lower set of blot tracks A) or anti-IR β-subunit (lower set of blot tracks B). These blots also indicate equal protein loading.

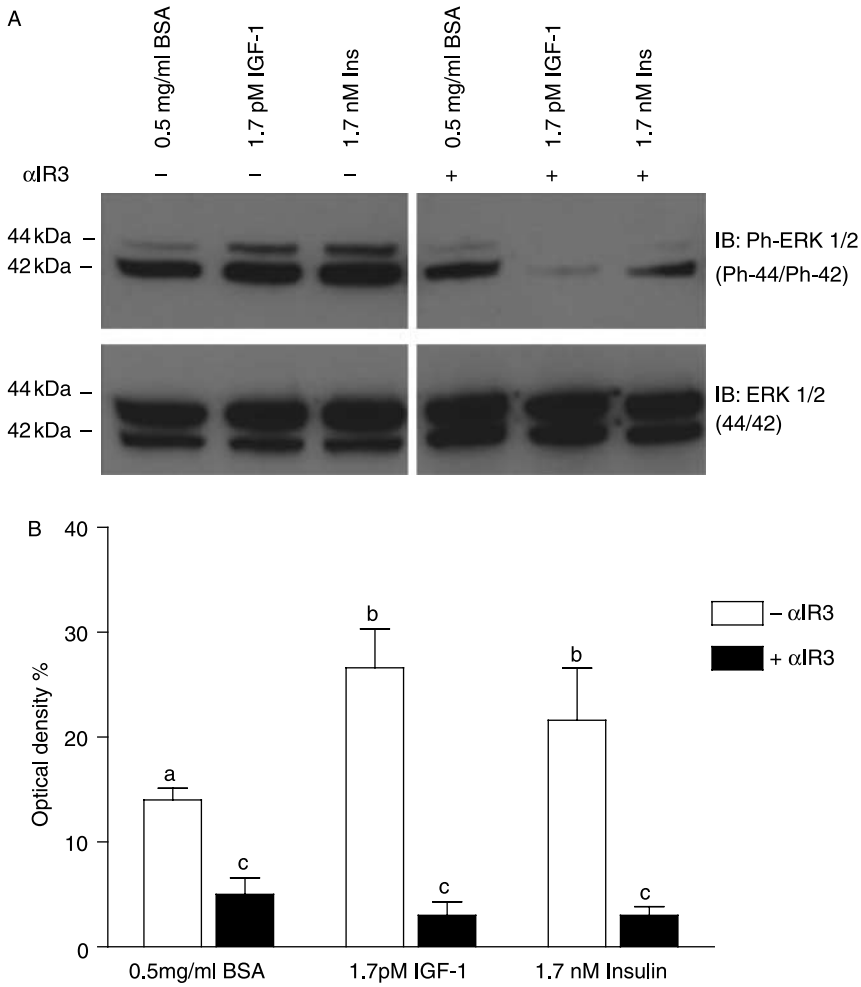

Figure 5 IGF-I or insulin cause phosphorylation of MAPK/ERK1/2 in mES cells. (A) Phosphorylation of ERK1/2 in response to 1.7 pM IGF-I, 1.7 nM insulin and in control medium with 0.5 g BSA l^{-1}. After stimulation, lysates were immunoblotted with anti-phospho ERK1/2 antibody (Ph-ERK1/2). The phosphorylation of ERK1/2 was increased by both 1.7 pM IGF-I and 1.7 nM insulin, but this was reduced by αIR3. αIR3 also blocked phosphorylation of ERK1/2 in the basal medium containing only 0.5 g BSA l^{-1}. The lower panel shows the same membrane after stripping, and reprobing with anti-ERK1/2 shows equal loading of the protein in the gel and for comparative densitometric analysis. (B) Quantitative scans show the ratio of the optical density of the phospho-ERK1/2 band as a percentage of the band stripped and blotted with the ERK1/2 antibody, mean (±S.E.M., $n=3$). Note similar levels of phosphorylation of ERK1/2 initiated by IGF-I and insulin and a significantly lower level of ERK1/2 activation in BSA control media ($P<0.05$, $n=3$, ANOVA, 'a' versus 'b'). αIR3 (black bars), significantly reduced phosphorylation of ERK1/2 initiated by all three treatments ($P<0.05$, $n=3$, ANOVA, 'a' and 'b' versus 'c').

The α-subunit demonstrates an apparent molecular mass of ∼135 kDa and the β-subunit, ∼95 kDa. R1ES cells expressed an IGF-1R α-subunit doublet, which was also apparent for IR α-subunit, of apparent molecular mass 130–132 kDa and β-subunit complex with major bands equivalent to ∼97 and 105 kDa, whereas only the 105 kDa isoform was detected in E18 skeletal muscle. The IR did not present the higher mobility band. Multiple forms of the IGF-1R subunits have been attributed to different post-translational processes (Alexandrides & Smith 1989) and are commonly observed in rapidly growing tissues such as neuroblastoma (Ota *et al.* 1988*a*, 1988*b*). An IGF-1R β-subunit isoform of 105 kDa was reported in fetal rat fibroblasts (Alexandrides & Smith 1989, Moss & Livingston 1993, Seely *et al.* 1995) and other highly proliferative cells (Belfiore *et al.* 1999; Garcia-de Lacoba *et al.* 1999; Pandini *et al.* 1999), suggesting that expression of this form is associated with a highly proliferative state. Alternatively, the 105 kDa IGF-1R β-subunit may hybridise with the IR (Seely *et al.* 1995) to form IR–IGF-1R hybrid receptors. Such hybrid receptors have been observed in most mammalian cells (Moxham *et al.* 1989, Soos *et al.* 1993, Bailyes *et al.* 1997), but have not been described in ES cells or preimplantation embryos.

IGF-I and insulin cause a dose-dependent increase in protein synthesis in mES cells

The 100-fold different potencies of IGF-I and insulin on R1ES cell protein synthesis match the affinities from competitive binding assays using mES cells (Shi *et al.* 1995). As further confirmation, a blocking antibody (αIR3) completely abolished the protein synthetic stimulus from both ligands and significantly attenuated IGF-1R autophosphorylation, supporting the conclusion that insulin and IGF-I stimulate ES cell protein synthesis through activation of the IGF-1R and not the IR. Indeed, neither ligand activated the IR. This contrasts with studies of mouse blastocysts, where reversed potencies indicated that both ligands acted through IR, not IGF-1R to acutely stimulate protein synthesis (Harvey & Kaye 1991*a*). The most likely explanation for the different mediating receptors of R1ES cells and blastocysts is that the current

study measured the response of ES cells derived exclusively from the ICM, whilst the study by Harvey & Kaye (1991*a*) examined whole blastocysts. Since in the blastocyst, the epithelial trophectoderm (TE) cells completely envelop the ICM, these earlier data may reflect a phenotype of TE cells rather than ICM cells, which could be expected to be modelled by ES cells. However, in whole blastocysts, IGF-I increases ICM proliferation (Harvey & Kaye 1992*b*) and inhibits apoptosis (Spanos *et al.* 2000); these effects may also - simultaneously increase protein synthesis of ICM cells as observed in this study of ES cells. The fact that IGF-I stimulates growth of mES cells suggests benefits to ES growth from adding this growth factor to defined media.

IGF-I and insulin activation of IGF-1R leads to activation of the MAPK pathway

Phosphorylation of the IGF-1R activates several intracellular signalling pathways that ultimately modulate diverse physiological parameters, including metabolism and cell proliferation (Adams *et al.* 2004, Larsson *et al.* 2005). Our studies show that binding of either IGF-I or insulin activates the IGF-1R tyrosine kinase leading to phosphorylation of MAPK ERK1/2 in R1ES cells. This pathway is generally associated with the growth and proliferative outcomes of activation of the IGF-1R, suggesting that the stimulation of protein synthesis observed in ES cells was a consequence of increased cell proliferation. These observations are supported by studies of rabbit blastocysts that similarly showed insulin-induced ERK1/2 phosphorylation in the absence of effects on glucose transport (Navarrete Santos *et al.* 2004). Inhibition of FCS-induced MEK/ERK phosphorylation in mES cells with U0126 had no effect on apoptosis, but there was no assessment of cell proliferation, which is commonly associated with MAPK/ERK activation (Gross *et al.* 2005).

In conclusion, both IGF-1R and IR are expressed in mES cells, as in progenitor ICM cells. However, in contrast to blastocysts, the IGF-1R is the mediating receptor for both ligands in mES cells, and its activation leads to activation of ERK1/2. The lack of mES cell IR phosphorylation by either agonist questions the physiological role of IR in mES cells.

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References

Adams TE, Mckern NM & Ward CW 2004 Signalling by the type 1 insulin-like growth factor receptor: Interplay with the epidermal growth factor receptor. *Growth Factors* **22** 89–95.

Alexandrides TK & Smith RJ 1989 A novel fetal insulin-like growth factor (IGF) I receptor. Mechanism for increased IGF I- and insulin-stimulated tyrosine kinase activity in fetal muscle. *Journal Biological Chemistry* **264** 12922–12930.

Avruch J 1998 Insulin signal transduction through protein kinase cascades. *Molecular and Cellular Biochemistry* **182** 31–48.

Bailyes EM, Nave BT, Soos MA, Orr SR, Hayward AC & Siddle K 1997 Insulin receptor/IGF-I receptor hybrids are widely distributed in mammalian tissues: quantification of individual receptor species by selective immunoprecipitation and immunoblotting. *Biochemical Journal* **327** 209–215.

Belfiore A, Pandini G, Vella V, Squatrito S & Vigneri R 1999 Insulin/IGF-I hybrid receptors play a major role in IGF-I signaling in thyroid cancer. *Biochimie* **81** 403–407.

Chi MM, Schlein AL & Moley KH 2000 High insulin-like growth factor 1 (IGF-1) and insulin concentrations trigger apoptosis in the mouse blastocyst via down-regulation of the IGF-1 receptor. *Endocrinology* **141** 4784–4792.

Garcia-de Lacoba M, Alarcon C, de la Rosa EJ & de Pablo F 1999 Insulin/insulin-like growth factor-I hybrid receptors with high affinity for insulin are developmentally regulated during neurogenesis. *Endocrinology* **140** 233–243.

Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP & Sanes JR 1996 Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* **85** 525–535.

Gross VS, Hess M & Cooper GM 2005 Mouse embryonic stem cells and preimplantation embryos require signaling through the phosphatidylinositol 3-kinase pathway to suppress apoptosis. *Molecular Reproduction and Development* **70** 324–332.

Harvey MB & Kaye PL 1988 Insulin stimulates protein synthesis in compacted mouse embryos. *Endocrinology* **122** 1182–1184.

Harvey MB & Kaye PL 1990 Insulin increases the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts *in vitro*. *Development* **110** 963–967.

Harvey MB & Kaye PL 1991*a* Mouse blastocysts respond metabolically to short-term stimulation by insulin and IGF-1 through the insulin receptor. *Molecular Reproduction and Development* **29** 253–258.

Harvey MB & Kaye PL 1991*b* Visualization of insulin receptors on mouse pre-embryos. *Reproduction Fertility and Development* **3** 9–15.

Harvey MB & Kaye PL 1992*a* Mediation of the actions of insulin and insulin-like growth factor-1 on preimplantation mouse embryos *in vitro*. *Molecular Reproduction and Development* **33** 270–275.

Harvey MB & Kaye PL 1992*b* Insulin-like growth factor-1 stimulates growth of mouse preimplantation embryos *in vitro*. *Molecular Reproduction and Development* **31** 195–199.

Hogan B, Beddington R, Costantini F & Lacy E 1994 *Manipulating the Mouse Embryo: A Laboratory Manual*, Spring Harbour, New York: Cold Spring Harbour Laboratory Press.

Kaye PL 1997 Preimplantation growth factor physiology. *Reviews of Reproduction* **2** 121–127.

Kaye PL, Bell KL, Beebe LF, Dunglison GF, Gardner HG & Harvey MB 1992 Insulin and the insulin-like growth factors (IGFs) in preimplantation development. *Reproduction Fertility and Development* **4** 373–386.

Kull FC Jr, Jacobs S, Su YF, Svoboda ME, Van Wyk JJ & Cuatrecasas P 1983 Monoclonal antibodies to receptors for insulin and somatomedin-C. *Journal Biological Chemistry* **258** 6561–6566.

Larsson O, Girnita A & Girnita L 2005 Role of insulin-like growth factor 1 receptor signalling in cancer. *British Journal of Cancer* **92** 2097–2101.

Markham KE & Kaye PL 2003 Growth hormone, insulin-like growth factor I and cell proliferation in the mouse blastocyst. *Reproduction* **125** 327–336.

Moss AM & Livingston JN 1993 Distinct beta-subunits are present in hybrid insulin-like-growth-factor-1 receptors in the central nervous system. *Biochemical Journal* **294** 685–692.

Moxham CP, Duronio V & Jacobs S 1989 Insulin-like growth factor I receptor beta-subunit heterogeneity, Evidence for hybrid tetramers composed of insulin-like growth factor I and insulin receptor heterodimers. *Journal Biological Chemistry* **264** 13238–13244.

Nagy A, Rossant J, Nagy R, Abramow-Newerly W & Roder JC 1993 Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *PNAS* **90** 8424–8428.

Navarrete Santos A, Tonack S, Kirsten M, Pantaleon M, Kaye PL & Fischer B 2004 Insulin acts via mitogen-activated protein kinase phosphorylation in rabbit blastocysts. *Reproduction* **128** 517–526.

Ota A, Wilson GL, Spilberg O, Pruss R & LeRoith D 1988*a* Functional insulin-like growth factor I receptors are expressed by neural-derived continuous cell lines. *Endocrinology* **122** 145–152.

Ota A, Wilson GL & Leroith D 1988*b* Insulin-like growth factor I receptors on mouse neuroblastoma cells. Two beta subunits are derived from differences in glycosylation. *European Journal of Biochemistry* **174** 521–530.

Pandini G, Vigneri R, Costantino A, Frasca F, Ippolito A, Fujita-Yamaguchi Y, Siddle K, Goldfine ID & Belfiore A 1999 Insulin and insulin-like growth factor-I (IGF-I) receptor overexpression in breast cancers leads to insulin/IGF-I hybrid receptor overexpression: evidence for a second mechanism of IGF-I signaling. *Clinical Cancer Research* **5** 1935–1944.

Patton BL, Cunningham JM, Thyboll J, Kortesmaa J, Westerblad H, Edstrom L, Tryggvason K & Sanes JR 2001 Properly formed but improperly localized synaptic specializations in the absence of laminin alpha4. *Nature Neuroscience* **4** 597–604.

Rechler MM & Nissley SP 1985 The nature and regulation of the receptors for insulin-like growth factors. *Annual Reviews* **47** 425–442.

Robertson EJ 1987 *Teratocarcinomas and Embryonic Stem Cells a Practical Approach,* Washington DC: IRL Press.

Rohlik QT, Adams D, Kull FC Jr & acobs S 1987 An antibody to the receptor for insulin-like growth factor I inhibits the growth of MCF-7 cells in tissue culture. *Biochemical and Biophysical Research Communications* **149** 276–281.

Rother KI & Accili D 2000 Role of insulin receptors and IGF receptors in growth and development. *Pediatric Nephrology* **14** 558–561.

Seely BL, Reichart DR, Takata Y, Yip C & Olefsky JM 1995 A functional assessment of insulin/insulin-like growth factor-I hybrid receptors. *Endocrinology* **136** 1635–1641.

Shi CZ, Dhir RN, Kesavan P, Zhang SL, Matschinsky FM & Heyner S 1995 Mouse embryonic stem cells express receptors of the insulin family of growth factors. *Molecular Reproduction and Development* **42** 173–179.

Soos MA, Field CE & Siddle K 1993 Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity. *Biochemical Journal* **290** 419–426.

Spanos S, Becker DL, Winston RM & Hardy K 2000 Anti-apoptotic action of insulin-like growth factor-I during human preimplantation embryo development. *Biology of Reproduction* **63** 1413–1420.

Taniguchi CM, Emanuelli B & Kahn CR 2006 Critical nodes in signalling pathways: insights into insulin action. *Nature Reviews Molecular Cellular Biology* **7** 85–96.

Wood SA, Allen ND, Rossant J, Auerbach A & Nagy A 1993 Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* **365** 87–89.

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