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ORIGINAL RESEARCH

The IGF system in *in-vitro* human decidualization

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ABSTRACT: Decidualization of endometrial stromal cells (ESCs) is critical for a successful pregnancy but the molecular mechanisms of the process are poorly understood. In this study, we investigated whether the insulin-like growth factor (IGF) network is involved in this cellular process. Expression kinetics of members of the IGF system was examined at both mRNA and protein levels during *in-vitro* decidualization of cultured human ESCs. We found a significant up-regulation of IGF-II as well as of IGF-I receptor and the A and B insulin receptor (InsR) isoforms. In addition, levels of the key adaptor proteins insulin receptor substrate 1 (IRS-1) and IRS-2 increased, suggesting a potential involvement of the IGF signalling pathway in the decidualization process. Expression of two IGF binding proteins, IGFBP-1 and IGFBP-4, which can inhibit IGF action, also increased. In order to determine whether IGF signalling was activated during decidualization, the phosphorylation status of the receptors and the adaptor proteins was estimated. Only IRS-2 was slightly phosphorylated in decidualized cells and was further activated by the addition of exogenous IGF-II. These results suggest that the IGF signalling pathway could play a crucial role in the functions of decidualized endometrial cells.

Key words: cAMP / decidualization / endometrium / insulin-like growth factor / progesterone

Introduction

Decidualization of endometrial stromal cells (ESCs) is crucial for a successful placentation and for the maintenance of pregnancy. It has been demonstrated that inadequate uterine decidualization could compromise female fertility (Benson *et al.*, 1996; Robb *et al.*, 1998; Lim *et al.*, 1999; Karpovich *et al.*, 2005) or promote trophoblast cell invasion resulting in intramyometrial placentation (Goffin *et al.*, 2003b).

The decidualization process consists of morphological and functional differentiation of ESCs occurring during the late secretory phase of the menstrual cycle, with the process progressing if pregnancy arises (Ferenczy and Bergeron, 1991). Decidualization can be induced *in-vitro* in ESCs by progesterone after estradiol priming or by different cAMP inducers such as prostaglandin E_2, relaxin and gonadotrophins (LH, FSH) (Tang and Gurpide, 1993; Frank *et al.*, 1994; Lane *et al.*, 1994; Brar *et al.*, 1997). Decidualization is induced with maximal efficiency by a combination of 8-bromo-cAMP (8-br-cAMP) and medroxyprogesterone acetate (MPA) (Gellersen and Brosens, 2003).

Insulin-like growth factors I (IGF-I) and II (IGF-II) act as endocrine, paracrine and autocrine factors and are implicated in various cellular activities such as proliferation, survival and differentiation. Their actions are mediated by type I IGF receptor (IGF-IR) and insulin receptor (InsR), which are highly homologous members of the tyrosine kinase receptor family (Ullrich *et al.*, 1986). Both IGF-IR and InsR are tetrameric receptors composed of two extracellular α-subunits containing the ligand binding domain, and two β-subunits which contain the tyrosine kinase domain. Human InsR exists in two isoforms, A (InsR-A) and B (InsR-B), generated from the alternative splicing of exon 11, coding for the C-terminus of the α-subunit. The expression of both isoforms is tightly regulated in a tissue-specific manner (Moller *et al.*, 1989; Mosthaf *et al.*, 1990), likely reflecting distinct functions. Moreover, it has been shown that InsR-A, but not InsR-B, binds to IGF-II with a high affinity, leading to increased mitogenic effects (Frasca *et al.*, 1999). Besides the classical receptors, hybrid IGF-I/Ins receptors are present in mammalian tissues (Bailyes *et al.*, 1997; Federici *et al.*, 1997). Functional studies have shown that hybrid receptors behave in a manner similar to IGF-IR since they bind to IGF-I with a higher affinity than to insulin (Soos *et al.*, 1993; Seely *et al.*, 1995). Upon ligand binding, the activated receptors recruit and phosphorylate scaffold proteins, such as insulin receptor substrate 1 (IRS-1) and IRS-2, leading to the activation of several intracellular signalling pathways including the phosphatidylinositol-3 kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (Siddle *et al.*, 2001; White, 2002). The type II IGF receptor (IGF-IIR) binds almost exclusively IGF-II and is considered as a scavenger receptor for IGF-II. It is unclear whether the receptor is able to initiate a signalling cascade.

The IGF network also includes six high-affinity IGF binding proteins (IGFBPs) regulating the interaction of IGFs with their receptors. IGFBP

modulation of IGF action is complex, precluding the understanding of the consequences of different IGFBP levels on IGF functions (Firth and Baxter, 2002). Due to their high affinity for IGFs, IGFBPs are able to prevent the binding of the ligands to their receptors. Post-translational modifications of some IGFBPs, as well as interaction with extracellular matrix components, affect their affinity for IGFs and therefore modulate IGF actions. In particular, proteolysis represents a major process in the regulation of IGF bioavailability. IGFBP-2 to -5 are proteolysis targets. IGFBP-3 and -5 proteolytic fragments conserve significant residual affinity for IGFs but IGFBP-2 and IGFBP-4 fragments have a drastically reduced IGF binding capacity (Jones and Clemmons, 1995).

Several observations have emphasized a potential role for the IGF network in ESC differentiation. IGF network expression is modulated in the endometrium and decidua during the normal menstrual cycle, under the influence of steroid hormones (Nayak and Giudice, 2003). Moreover, several microarray analyses have shown regulation of various members of the IGF network in ESC cultures which have been induced to differentiate (Brar *et al*., 2001; Tierney *et al*., 2003; Giudice, 2004).

In this study, we describe the expression of members of the IGF network during *in-vitro* differentiation of the stromal cells induced by the combination of cAMP and MPA. We provide detailed kinetics of the expression of the ligands (IGF-I and IGF–II), the IGF binding proteins (IGFBP-1 to -6), the receptors (InsR, IGF-IR and IGF-IIR) and the key adaptor proteins (IRS-1 and IRS-2). We found that decidualization was accompanied by an increase in IGF-II, IGFBP-1 and IGFBP-4 and by a decrease in IGFBP-5. Concerning the receptors, the expression of both InsR isoforms and of IGF-IR was up-regulated by the decidualizing treatment. Expression levels of IRS-1 and IRS-2 increased equally. To investigate whether the IGF signalling pathway was activated during ESC differentiation, we also examined phosphorylation of the receptors and the adaptor proteins in decidualized cells with or without the addition of exogenous IGF peptides.

Materials and Methods

Tissue acquisition

Endometrial biopsies were obtained from premenopausal, cycling and not hormonally treated women (age range: 31–42 years). The patients were examined for benign reasons with the authorization of the local ethical committee and had given their written informed consent. A portion of each endometrial specimen was fixed in formalin for histological dating according to the criteria of Noyes *et al*. (1975).

Isolation and culture of ESCs

Normal proliferative and early secretory endometrial biopsies (between days 6 and 16 of the menstrual cycle) were collected in HBSS containing 1% penicillin-streptomycin (Cambrex Bio Science, Baltimore, MD, USA). ESCs were isolated according to the method of Brosens *et al*. (1999) with minor modifications. Briefly, endometrial tissue was finely minced and enzymatically digested with 0.2% collagenase (Sigma, St Louis, MO, USA) and 100 IU/ml deoxyribonuclease type I (Roche Molecular Biochemicals, Mannheim, Germany) for 45 min at 37°C. After centrifugation at 1000*g* for 5 min, the pellets were suspended in DMEM/F12 (Gibco BRL/Invitrogen, Carlsbad, CA, USA) containing 10% charcoal-stripped FBS (Cambrex Bio Science), 1% penicillin–streptomycin and 0.25 μg/ml fungizone (Gibco BRL/Invitrogen). Cells were sedimented by gravitation for 30 min to separate stromal cells from glands. The ESC fraction was enriched by the more rapid adherence of stromal cells to the dish and was further separated from contaminating epithelial cells by selective trypsinization. After two passages, the purity of stromal cell cultures was assessed by negative cytokeratin immunostaining (<5% of epithelial cells).

Cells from passage 2 were grown in 10% charcoal-stripped FBS DMEM/F12 medium until they reached confluence and were then switched to medium containing 2% charcoal-treated FBS for 24 h. Decidualizing treatment consisting of 1 μM MPA (Sigma) and 0.5 mM 8-br-cAMP (Sigma) was applied for the indicated time periods. The culture medium was renewed every 3 days. Differentiation was assessed by examination of cell morphology under phase contrast microscopy at various times during the treatment and by measuring the expression of decidualization-specific marker genes, namely IGFBP-1 and prolactin (PRL).

RNA extraction and RT–PCR

Total RNA from ESC cultures was isolated using TriPure (Roche Molecular Biochemicals) reagent according to the manufacturer's instructions and was treated with RNase-free DNase (Roche Molecular Biochemicals). First-strand cDNA was synthesized from 250 ng total RNA using random hexamers (Invitrogen) and AMV Reverse Transcriptase (Promega, Madison, WI, USA) in a final volume of 50 μl. For cDNA amplification, a 2 μl aliquot of reverse transcription product was amplified in a volume of 25 μl, containing 0.5 mM $MgCl_2$, 200 μM of each deoxynucleotide, 0.2 μM of each primer and 2.5 IU *Taq* DNA polymerase (Perkin-Elmer Life Sciences, Boston, MA, USA). After denaturation at 95°C for 2 min, PCR amplification cycles consisted of 30 s at 94°C, 60 s at the annealing temperature and 90 s at 72°C, followed by a final 7 min extension at 72°C. The sequences of the oligonucleotide primers (Eurogentec, Seraing, Belgium) and their respective annealing temperature used for PCR reactions are given in Table I. The PCR products were quantified after electrophoresis on a 10% polyacrylamide gel and staining with Gelstar (FMC Bioproducts, Rockland, ME, USA) using a Fluor-S™ Multilmager (Bio-Rad Laboratories, Hercules, CA, USA). Densitometric values of each assay sample were normalized with 28S rRNA levels used as the internal control.

Protein extraction and western blot analysis

Cells were scraped and homogenized in lysis buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 1 mM EGTA, 10% glycerol, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 100 mM NaF, protease inhibitor cocktail Complete Mini (Roche Molecular Biochemicals)]. The protein concentration of the lysates was determined using Pierce BCA Protein Assay Reagent kit (Pierce, Rockford, IL, USA). Samples of 30 μg protein per lane were resolved on a reducing 10–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and were electroblotted onto a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked by soaking in Tween Tris buffer [0.1% Tween 20, 50 mM Tris (pH 8.0), 150 mM NaCl] containing 5% non-fat dried milk for 1 h and was then incubated with diluted primary antibody for 2 h at room temperature. The primary antibodies used were rabbit polyclonal antibodies for IGF-IR (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), InsR (dilution 1:1000; Santa Cruz Biotechnology), IRS-1 (dilution 1:1000; Santa Cruz Biotechnology), IRS-2 (dilution 1:500; Santa Cruz Biotechnology) and pregnancy associated plasma protein-A (PAPP-A)/pro-MBP complex (dilution 1:1000; DakoCytomation, Glostrup, Denmark), mouse monoclonal antibodies for IGBP-4 (dilution 1:1000; Austral Biologicals, San Ramon, CA, USA) and IGFBP-5 (dilution 1:500; Santa Cruz Biotechnology), goat polyclonal antibodies for IGFBP-1 (dilution 1:1000; Santa Cruz Biotechnology) and Ku70 (dilution 1:1000; Santa Cruz Biotechnology) which was used as the protein loading control. Signals were detected after incubation with horseradish

Table I Sequences of the oligonucleotides used in the PCR reactions

Primer		Sequence (5′–3′)	Annealing temperature (°C)	Reference
IGF-I	Forward	TGGATGCTCTTCAGTTCGTG	59	
	Reverse	CTGACTTGGCAGGCTTGAG		
IGF-II	Forward	CGTCGCAGCCGTGGCATCGTTGA	57	
	Reverse	GCCCACGGGGTATCTGGGGAAGT		
IGFBP-1	Forward	AGACGACGGAGATAACTGAGGA	57	
	Reverse	GCCTTGGCTAAACTCTCTACGA		
IGFBP-2	Forward	CGGAAGCCCCTCAAGTCG	57	(Yi *et al.*, 2001)
	Reverse	GCCTCCTGCTGCTCATTG		
IGFBP-3	Forward	CTCTCCCAGGCTACACCA	57	(Yi *et al.*, 2001)
	Reverse	GAAGTCTGGGTGCTGTGC		
IGFBP-4	Forward	AAATTCGAGACCGGAGCAC	59	
	Reverse	AGCTTCACCCCCGTCTTC		
IGFBP-5	Forward	AAGAAGGACCGCAGAAAG	57	(Yi *et al.*, 2001)
	Reverse	GGGGACGCATCACTCAAC		
IGFBP-6	Forward	AACCGCAGAGACCAACAGAG	55	
	Reverse	TTGGGCACGTAGAGTGTTTG		
InsR	Forward	TTCCGAGACCTCAGTTTC	55	(Wu *et al.*, 2003)
	Reverse	TGTGACTTACAGATGGT		
InsRexon11	Forward	AACCAGAGTGAGTATGAGGAT	58	(Frasca *et al.*, 1999)
	Reverse	CCGTTCCAGAGCGAAGTGCTT		
IGF-IR	Forward	GTGTACGTTCCTGATGAGTGGGAG	65	(Roy *et al.*, 1997)
	Reverse	GCCCCGTGTCATCAGTTCCATGAT		
IGF-IIR	Forward	TCAACATCTGTGGAAGTGTG	57	(Ilvesmaki *et al.*, 1993)
	Reverse	GAATAGAGAAGTGTCCGGATCGGAGTC		
IRS-1	Forward	CTGGACATCACAGCAGAATGA	59	
	Reverse	CGCTTGGCACAATATAGAACG		
IRS-2	Forward	AGATCTGTCTGGCTTTATCACCAGGA	55	(Wu *et al.*, 2003)
	Reverse	CCTAGCATGCTGAGGGTTATATCTGC		
PRL	Forward	GGTGACCCTTCGAGACCTGTT	60	(Goffin *et al.*, 2003a)
	Reverse	GGAAGAAGTGTGGCAGCTGTT		
28S	Forward	GTTCACCCACTAATAGGGAACGTGA	68	(Goffin *et al.*, 2003a)
	Reverse	GATTCTGACTTAGAGGCGTTCAGT		

The primers where no reference is indicated were designed by using Primer3 program (Rozen and Skaletsky, 2000).

peroxidase-conjugated secondary antibody (dilution 1:2000; DakoCytomaton) by using the ECL Western blotting system (Amersham Biosciences, Buckinghamshire, UK).

Preparation and analysis of conditioned medium from cell culture

Conditioned medium (CM) was collected after 8 h, 1, 3, 6, 9 and 12 days and was centrifuged at 12 000*g* for 10 min to remove debris. The harvested CM was concentrated 5-fold by lyophilization and stored at −70°C. IGF-II secreted into the CM was quantified by immunoradiometric assay (IRMA) (Diagnostic Systems Laboratories Inc., Webster, TX, USA) according to the manufacturer's instructions. The sensitivity of the IGF-II assay was 0.13 ng/ml and the intra- and inter-assay coefficients of variation were 4.3–7.2 and 6.3–10.4%, respectively. Levels of IGF-II for each sample at each time point were assayed in duplicate and were normalized to the amount of total protein in each well. Levels of secreted IGFBP-4, IGFBP-5 and PAPP-A were evaluated by immunoblotting.

Immunoneutralization of PAPP-A protease

Confluent ESC cultures were treated with cAMP and MPA or with ethanol carrier solution as the control (see above), in the absence or presence of polyclonal anti-PAPP-A/pro-MBP complex antibody (10 and 50 μg/ml) and control rabbit IgG (50 μg/ml) for 24 h. Proteolysis of endogenous IGFBP-4 in concentrated CM was analysed by western blot.

Phosphorylation of IGF-IR, InsR, IRS-1 and IRS-2

Phosphorylation of IGF-IR, InsR, IRS-1 and IRS-2 was studied in ESCs grown at confluence in 6 cm tissue culture dishes and treated with

cAMP and MPA or with ethanol for 6 days. Untreated and treated cells were stimulated with or without either 500 ng/ml IGF-II (Sigma) or Long R3 IGF-I (LR3 IGF-I) (GroPep Ltd., Adelaide, Australia) for 15 min, immediately prior to protein extraction. Long R3 IGF-I is a recombinant peptide that does not bind to IGFBPs. Cells were rinsed in ice-cold PBS and were incubated in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 0.25% sodium-deoxycholate, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 100 mM NaF, protease inhibitor cocktail Complete Mini (Roche Molecular Biochemicals)] for 30 min on ice. Cell debris was eliminated by centrifugation at 12 000*g* for 15 min. Samples containing 400 μg protein extract were precleared for 60 min at 4°C with control rabbit IgG (Santa Cruz Biotechnology) and protein-A sepharose (Amersham Biosciences). After centrifugation, the supernatant was incubated at 4°C overnight with 2 μg of the adequate antibody or control rabbit IgG and then for 1 h with 30 μl of protein-A sepharose. Immunoprecipitates were washed three times and diluted in 2× Laemmli Buffer. Samples were boiled for 5 min before loading onto 6–8% SDS–PAGE. Western blots were performed as described earlier by using anti-phosphotyrosine 4G10 antibody (anti-PY) (Upstate Biotechnology, Lake Placid, NY, USA).

Data analysis

Three different endometrial biopsies were analysed for the RT–PCR and western blot experiments. From each biopsy, duplicate cultures were performed for all experiments. Concerning the RT–PCR quantifications, the ratio of densitometric values in decidualized versus non-decidualized cells was calculated at each time point. The *t*-test was performed on the ratio data averaged from the three independent samples (Statistica 7.1, Statsoft). $P < 0.05$ was considered as statistically significant.

Results

Induction of decidualization by cAMP and MPA

The morphology of the ESCs was examined under phase contrast microscopy at various times after treatment to confirm *in-vitro* decidualization. The untreated ESCs retained their fibroblastic spindle-shaped appearance throughout the culture periods. The morphology of the ESCs exposed to 8-br-cAMP and MPA started to change after 3 days of culture. ESCs acquired a phenotype characteristic of decidualization with a polygonal morphology and a more abundant cytoplasm. Stromal changes were complete after 6 days of treatment (Fig. 1A). The second method for assessing the decidualization consisted of the evaluation of the expression of decidualization markers. PRL and IGFBP-1 mRNA levels were induced as soon as 8 h after the beginning of treatment and reached their maximum at 3 days (Fig. 1B). Moreover, IGFBP-1 protein was detected by immunoblotting in cellular protein extracts and in CM of treated cells (Fig. 1C). IGFBP-1 expression increased rapidly from 8 h up to 3 days and then remained elevated, in agreement with mRNA results.

Kinetics of IGF ligand expression

Total RNA was extracted from cells cultured with or without MPA and 8-br-cAMP for 8 h, 1, 3, 6 and 12 days. The expression of IGF-I and -II mRNAs was analysed by semi-quantitative RT–PCR. IGF-I was not significantly regulated by cAMP and MPA (data not shown). Significant induction of IGF-II mRNA levels was observed after 1 day and reached a maximum of 2.6-fold, after 3 days of treatment (Fig. 2A and B; $P < 0.05$ at 1, 3, 6 and 12 days). A moderate IGF-II mRNA increase was also observed in untreated cells but the increase in IGF-II remained statistically higher in treated cells up to 12 days of culture. IGF-II protein concentration was measured by IRMA in CM, collected at different time points of treatment. Production of IGF-II was significantly higher in cells treated with 8-br-cAMP and MPA from 6 days onwards (Fig. 2C; $P < 0.05$ at 6, 9 and 12 days).

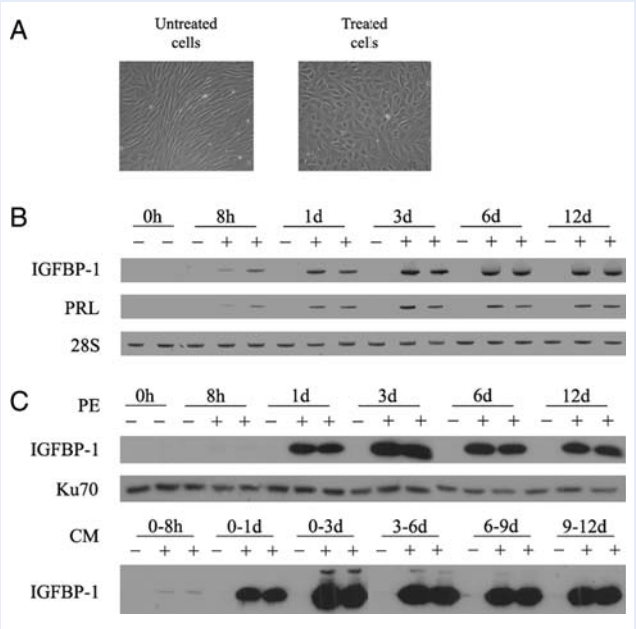
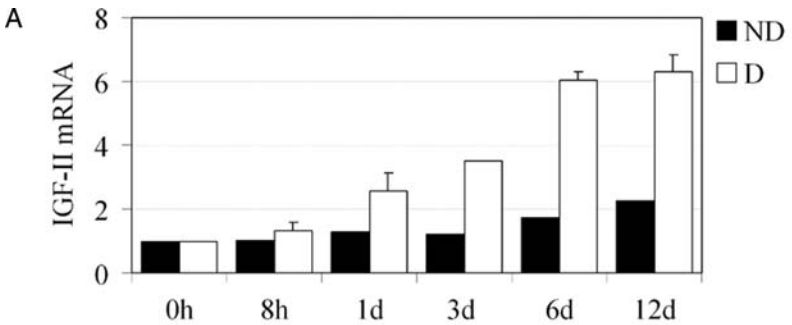


Figure 1 Assessment of *in-vitro* decidualization of ESCs. Confluent cultures of ESCs were treated with 0.5 mM 8-br-cAMP and 1 μM MPA or were cultured in control medium. The medium was renewed every 3 days. (**A**) Morphological appearance of ESCs under phase contrast microscopy after 6 days of culture. Cells treated with cAMP and MPA exhibited morphological changes typical of decidual cells with large polygonal form, while untreated cells retained a fibroblast-like spindle-shaped appearance (original magnification ×40). (**B**) Induction of IGFBP-1 and PRL mRNA levels in ESCs treated with cAMP and MPA. Total RNA was extracted after 0, 8 h, 1, 3, 6, 12 days of treatment from untreated cells (−) and from cells treated with cAMP and MPA (+). The expression of *IGFBP-1* and *PRL* genes was analysed by RT–PCR. One representative experiment of three similar experiments is shown. (**C**) IGFBP-1 protein levels measured by western blot. Immunoblotting of IGFBP-1 was performed on protein extracts (PE) and on conditioned media (CM) from untreated cells (−) and from cells treated with cAMP and MPA (+). Whole cell lysates were extracted after 0, 8 h, 1, 3, 6, 12 days of treatment and conditioned media were collected after 8 h (for period 0–8 h), 1 (for period 0–1 day), 3 (for period 0–3 days), 6 (for period 3–6 days), 9 (for period 6–9 days) and 12 (for period 9–12 days) days of culture. Experiments were repeated three times with similar results.

Kinetics of IGFBP expression

We compared the changes in IGFBP mRNA and protein levels in treated and untreated ESCs. As already mentioned, IGFBP-1



B

	0h	8h	1d	3d	6d	12d
IGF-II mRNA fold change	1	1.06+/-0.32	1.67+/-0.46*	2.60+/-0.51*	2.55+/-0.87*	1.98+/-0.64*

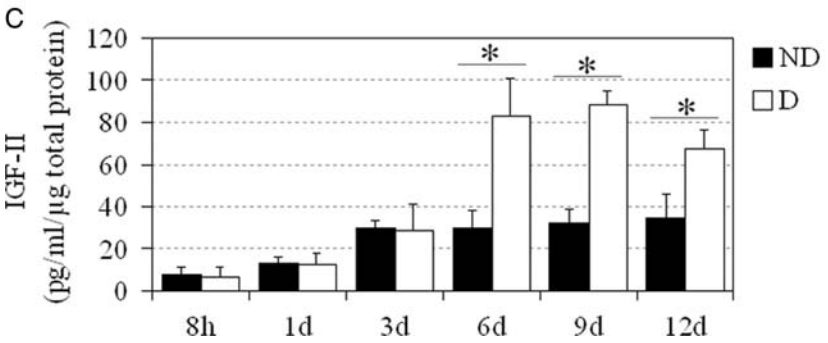
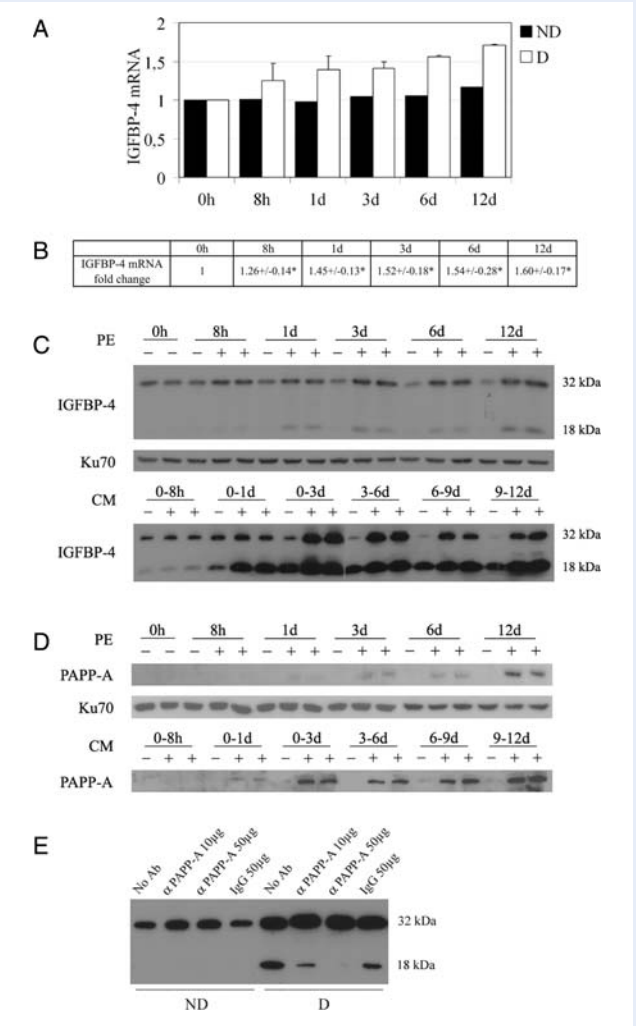


Figure 2 Expression time course of IGF-II during decidualization. (**A**) IGF-II mRNA levels measured by RT–PCR in ESC cultures. Total RNA was extracted from non-decidualized (ND) and decidualized (D) cells after 0, 8 h, 1, 3, 6 and 12 days of treatment. The presented results are from a single representative experiment. The densitometric values obtained for IGF-II mRNA at each time point were referred to the densitometric value at time 0 h considered as being equal to 1. (**B**) Fold induction of IGF-II mRNA levels. Table shows the ratio of densitometric values at each time point in decidualized versus non-decidualized cells. Data represent the mean and SD summarized from three independent experiments performed in duplicate. Statistical significance of the differences above value for time point 0 h was determined by the Student's *t*-test (*$P < 0.05$). (**C**) Production of IGF-II by cultured ESCs treated with cAMP and MPA. IGF-II concentration was determined by immunoradiometric assay in conditioned media of non-decidualized (ND) and decidualized (D) cells collected after 8 h (for period 0–8 h), 1 (for period 0–1 day), 3 (for period 0–3 days), 6 (for period 3–6 days), 9 (for period 6–9 days) and 12 (for period 9–12 days) days of culture. Concentration of IGF-II in DMEM/F12 2% charcoal-treated FBS was below the detection level. Each sample was assayed in duplicate and values were normalized to the total protein amount. Data represent the mean and SD of three separate experiments (*$P < 0.05$).

expression was induced by cAMP and MPA treatment (Fig. 1B and C). Among the five other IGFBPs, only the expression of IGFBP-4 and of IGFBP-5 was significantly altered by decidualizing treatment.

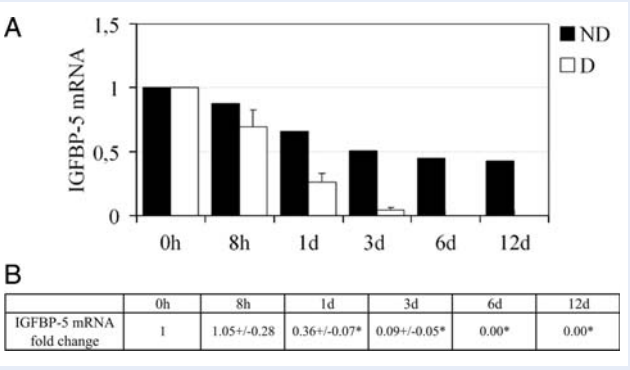
IGFBP-4 mRNA showed rapid and constant induction (Fig. 3A and B; $P < 0.05$ at 8 h, 1, 3, 6 and 12 days). The evolution of this protein level was followed both in cellular protein extracts and in CM of ESCs treated or not for decidualization, as the protein being secreted. As shown in Fig. 3C, cellular IGFBP-4 levels increased progressively starting from 8 h of decidualizing stimulation. A parallel evolution of the full length 32 kDa protein was observed in CM of treated cells. Moreover, a strong 18 kDa signal was detected in CM from both treated and untreated cells. This 18 kDa band could correspond to the proteolysis product of IGFBP-4 which has been identified in diverse cultured cell types.

In particular, the 18 kDa IGFBP-4 fragment has been described in CM of ESCs. The protease responsible for the degradation was identified as the PAPP-A (Giudice *et al.*, 2002). This protease belongs to the superfamily of zinc peptidases and circulates in pregnancy serum in complex with the precursor of major basic protein (pro-MBP) (Boldt *et al.*, 2001). To find out whether PAPP-A was responsible for IGFBP-4 proteolysis in CM from treated and untreated ESCs, we first investigated PAPP-A expression by immunoblotting with an anti-PAPP-A/pro-MBP complex antibody (Fig. 3D). The expression of the protease was increased by decidualizing treatment, as previously observed (Giudice *et al.*, 2002). Attribution of IGFBP-4 proteolysis to PAPP-A was assessed by immunoneutralization experiments. Cells were cultured for 24 h in the presence or absence of polyclonal anti-PAPP-A antibody and CM were subjected to IGFBP-4 western blot. As shown in Fig. 3E, addition of PAPP-A blocking antibody to the culture medium dose-dependently inhibited the formation of 18 kDa IGFBP-4 fragments in both untreated and treated cells, indicating that proteolysis of IGFBP-4 was due to PAPP-A.



	0h	8h	1d	3d	6d	12d
IGFBP-4 mRNA fold change	1	1.26+/-0.14*	1.45+/-0.13*	1.52+/-0.18*	1.54+/-0.28*	1.60+/-0.17*

Figure 3 Expression time course of IGFBP-4 during decidualization and involvement of the PAPP-A in the proteolysis of IGFBP-4. (**A**) IGFBP-4 mRNA levels measured by RT–PCR in ESC cultures. Total RNA was extracted from non-decidualized (ND) and decidualized (D) cells after 0, 8 h, 1, 3, 6 and 12 days of treatment. The presented results are from a single representative experiment. The densitometric values obtained for IGFBP-4 mRNA at each time point were referred to the densitometric value at time 0 h considered as being equal to 1. (**B**) Fold induction of IGFBP-4 mRNA levels. Table shows the ratio of densitometric values at each time point in decidualized versus non-decidualized cells. Data represent the mean and SD summarized from three independent experiments performed in duplicate. Statistical significance of the differences above value for time point 0 h was determined by the Student's *t*-test (*$P < 0.05$). (**C**) IGFBP-4 protein levels measured by western blot in ESC cultures. Immunoblotting of IGFBP-4 was performed on protein extracts (PE) and on conditioned media (CM) from untreated cells (−) and from cells treated with cAMP and MPA (+). Whole cell lysates were extracted after 0, 8 h, 1, 3, 6, 12 days of treatment and conditioned media were collected after 8 h (for period 0–8 h), 1 (for period 0–1 day), 3 (for period 0–3 days), 6 (for period 3–6 days), 9 (for period 6–9 days) and 12 (for period 9–12 days) days of culture. Experiments were repeated three times with similar results. (**D**) PAPP-A protein levels measured by western blot. Protein extracts (PE) and conditioned media (CM) from untreated cells (−) and from cells treated with cAMP and MPA (+) were analysed by western blot with polyclonal anti-PAPP-A/pro-MBP antibody. Whole cell lysates were extracted after 0, 8 h, 1, 3, 6, 12 days of treatment and conditioned media were collected after 8 h (for period 0–8 h), 1 (for period 0–1 day), 3 (for period 0–3 days), 6 (for period 3–6 days), 9 (for period 6–9 days) and 12 (for period 9–12 days) days of culture. Three separate experiments gave similar results. (**E**) Inhibition of the proteolysis of IGFBP-4 by PAPP-A immunoneutralization in ESC cultures. ESCs were decidualized with cAMP and MPA (D) or not (ND) in the presence of antibody against PAPP-A/pro-MBP complex (10 or 50 μg/ml) or non-specific anti-rabbit IgG (50 μg/ml). Conditioned media were collected after 24 h and were analysed by western blot by using anti-IGFBP-4 antibody.



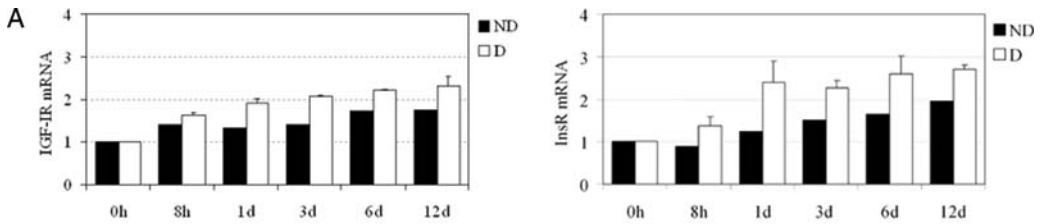
	0h	8h	1d	3d	6d	12d
IGFBP-5 mRNA fold change	1	1.05+/-0.28	0.36+/-0.07*	0.09+/-0.05*	0.00*	0.00*

Figure 4 Expression time course of IGFBP-5 during decidualization. (**A**) IGFBP-5 mRNA levels measured by RT–PCR in ESC cultures. Total RNA was extracted from non-decidualized (ND) and decidualized (D) cells after 0, 8 h, 1, 3, 6 and 12 days of treatment. The presented results are from a single representative experiment. The densitometric values obtained for IGFBP-5 mRNA at each time point were referred to the densitometric value at time 0 h considered as being equal to 1. (**B**) Fold induction of IGFBP-5 mRNA levels. Table shows the ratio of densitometric values at each time point in decidualized versus non-decidualized cells. Data represent the mean and SD summarized from three independent experiments performed in duplicate. Statistical significance of the differences above value for time point 0 h was determined by the Student's *t*-test (*$P < 0.05$).

IGFBP-5 mRNA expression was progressively and strongly inhibited in decidualized cells, becoming undetectable after 6 days of treatment (Fig. 4A and B; $P < 0.05$ at 1, 3, 6 and 12 days). Immunoblots of IGFBP-5 protein could not be reliably performed due to low levels of expression in stromal cells.

Kinetics of IGF and InsR expression

The changes in IGF-IR and InsR mRNA levels in cAMP and MPA treated cells compared with vehicle treated cells were evaluated by RT–PCR. The results, presented in Fig. 5A, show that the treatment stimulated IGF-IR and InsR expression. In contrast, IGF-IIR expression was unchanged (data not shown). Both IGF-IR and InsR mRNAs rapidly and gradually increased. The induction of IGF-IR mRNA levels peaked at 1.5-fold after 6 days of treatment (Fig. 5B; $P < 0.05$ at 8 h, 3, 6 and 12 days), whereas the induction of InsR



B

	0h	8h	1d	3d	6d	12d
IGF-IR mRNA fold change	1	1.19+/-0.12*	1.26+/-0.19	1.33+/-0.15*	1.53+/-0.15*	1.31+/-0.08*
InsR mRNA fold change	1	1.57+/-0.35*	2.00+/-0.23*	1.90+/-0.47*	1.59+/-0.25*	1.39+/-0.09*

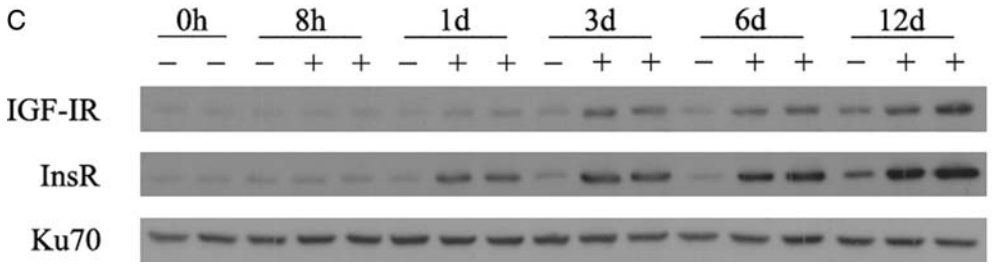


Figure 5 Expression time course of IGF-IR and InsR during decidualization. (**A**) IGF-IR and InsR mRNA levels measured by RT–PCR in ESC cultures. Total RNA was extracted from non-decidualized (ND) and decidualized (D) cells after 0, 8 h, 1, 3, 6 and 12 days of treatment. The presented results are from a single representative experiment. The densitometric values obtained for IGF-IR and InsR mRNA at each time point were referred to the densitometric value at time 0 h considered as being equal to 1. (**B**) Fold induction of IGF-IR and InsR mRNA levels. Table shows the ratio of densitometric values at each time point in decidualized versus non-decidualized cells. Data represent the mean and SD summarized from three independent experiments performed in duplicate. Statistical significance of the differences above value for time point 0 h was determined by the Student's *t*-test (*$P < 0.05$). (**C**) IGF-IR and InsR protein levels measured by western blot. Immunoblotting of InsR and IGF-IR was performed on protein extracts from untreated cells (−) and from cells treated with cAMP and MPA (+). Whole cell lysates were extracted after 0, 8 h, 1, 3, 6 and 12 days of culture. Experiments were repeated three times with similar results.

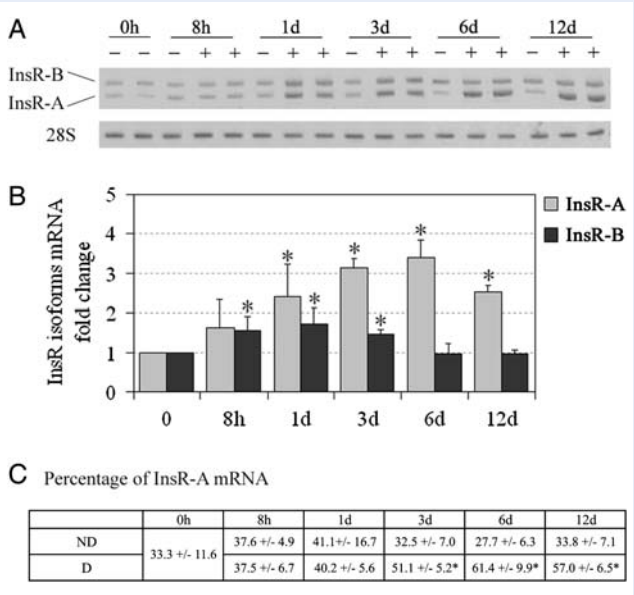
mRNA levels peaked at 2-fold after 1 day of treatment (Fig. 5B; $P < 0.05$ at 8 h, 1, 3, 6 and 12 days).

Next, we evaluated the modulation of IGF-IR and InsR protein levels by using specific antibodies for western blotting. In comparison to control cells, the levels of both receptors were slightly increased after 8 h of decidualizing treatment. A progressive increase in both receptor levels was observed throughout treatment (Fig. 5C). Levels of InsR and IGF-IR proteins also increased in control cell cultures, notably after 12 days of treatment, but the difference between decidualized and non-decidualized cells remained obvious at each time point.

Two InsR mRNA isoforms have been previously described, InsR-A and InsR-B, generated by the alternative splicing of exon 11 (Moller *et al*., 1989; Mosthaf *et al*., 1990). We investigated the relative abundance of both isoforms during decidualization, by RT–PCR using primers flanking exon 11. Results demonstrated that isoforms InsR-A and -B were expressed in untreated ESCs with the predominant expression of InsR-B (Fig. 6A). Both isoforms were affected by decidualizing treatment. The level of InsR-A rose to 3.4-fold after 6 days of treatment, while the InsR-B mRNA isoform level doubled after 1 day but decreased thereafter (Fig. 6B; $P < 0.05$ at 1, 3, 6 and 12 days for InsR-A and $P < 0.05$ at 8 h, 1 and 3 days for InsR-B). InsR-A represented ∼33% of the InsR mRNA present at the moment of treatment. Its relative percentage reached ∼51% after 3 days of treatment, becoming the major isoform from this time point onwards (Fig. 6C). This could lead to a significant IGF-II-mediated cell stimulation, since it has been shown that InsR-A binds IGF-II with high affinity (Frasca *et al*., 1999). In control cells, the relative proportion of the isoforms did not vary significantly.

Kinetics of IRS-1 and IRS-2

Levels of both IRS-1 and IRS-2 were significantly enhanced by the decidualizing treatment. IRS-1 mRNA increased rapidly, starting from 8 h of treatment, and peaked at 2.3-fold after 3 days of treatment (Fig. 7A and B; $P < 0.05$ at 8 h, 1, 3 and 6 days). A significant induction of IRS-2 mRNA was observed after 1 day in decidualized cells and reached a maximum of 2.1-fold after 3 days of treatment (Fig. 7A and B; $P < 0.05$ at 1, 3 and 6 days). The changes in IRS-1 and IRS-2 expression were confirmed at protein level by western blot. The specificity of the antibodies used to identify them was confirmed by the differential migration of the two proteins on SDS–PAGE, IRS-2 (∼180 kDa) migrating slower than IRS-1 (∼165 kDa). Both proteins levels started to increase from 8 h of treatment onwards (Fig. 7C). Concerning the IRS-2 immunoblot, we observed a diffuse signal in



	0h	8h	1d	3d	6d	12d
ND	33.3 +/- 11.6	37.6 +/- 4.9	41.1+/- 16.7	32.5 +/- 7.0	27.7 +/- 6.3	33.8 +/- 7.1
D		37.5 +/- 6.7	40.2 +/- 5.6	51.1 +/- 5.2*	61.4 +/- 9.9*	57.0 +/- 6.5*

Figure 6 Expression time course of InsR isoform mRNA during decidualization. (**A**) InsR isoform mRNA levels measured by RT–PCR in ESCs. Total RNA was extracted after 0, 8 h, 1, 3, 6, 12 days of treatment from untreated cells (−) and from cells treated with cAMP and MPA (+). PCR experiments were performed with primers flanking exon 11 of *InsR* gene to distinguish isoform-A (InsR-A; 600 bp) and -B (InsR-B; 636 bp). The figure presents a representative RT–PCR experiment of three independent experiments. (**B**) Fold induction of InsR isoform mRNA levels in ESC cultures. At each time point, results are presented as the ratio of densitometric values for InsR-A and InsR-B mRNA in decidualized versus non-decidualized cells. Means and SD are calculated from three independent experiments performed in duplicate. Statistical significance of the differences above value for time point 0 h was determined by the Student's *t*-test (*$P < 0.05$). (**C**) Relative abundance of InsR-A mRNA during decidualization. Table presents the relative percentage of InsR-A mRNA in untreated cells (ND) and in cells treated with cAMP and MPA (D). Each value is the mean ± SD of three separate experiments performed in duplicate.

decidualized cells, with slightly faster electrophoretic mobility in comparison with the signal in non-decidualized cells. This could be due to some degree of serine dephosphorylation since IRS-2 is a serine-rich protein (White, 2002).

Phosphorylation status of IGF-IR, InsR, IRS-1 and IRS-2 in decidualized cells

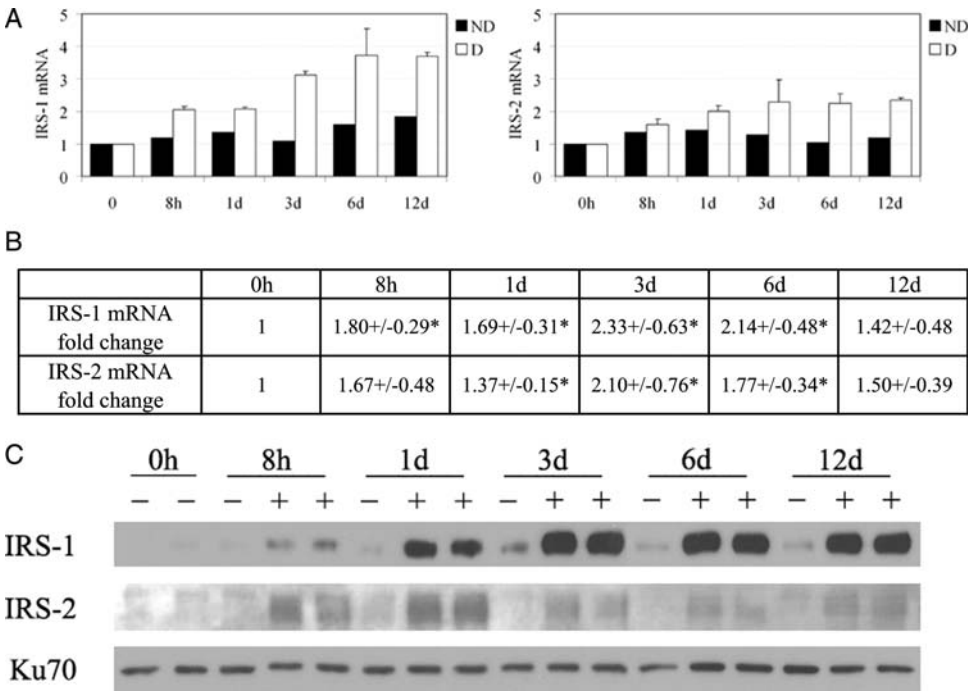
The up-regulation of IGF-II, IGF-IR and InsR as well as of IRS-1 and -2 during the time course of decidualization is consistent with an autocrine/paracrine activation of the IGF signalling pathway. To verify this hypothesis, we investigated the tyrosine phosphorylation levels of the receptors and the adaptor proteins, after 6 days of decidualizing treatment, in the absence or presence of exogenous IGFs. Proteins were immunoprecipitated using specific antibodies for each receptor and adaptor protein and were then probed with an anti-phosphotyrosine antibody (αPY). Western blots using respective antibodies confirmed equal immunoprecipitation of proteins between basal and IGF-stimulated conditions (data not shown). In the absence of exogenous IGFs, IGF-IR and InsR were barely phosphorylated, since faint signals were detected only after a long exposure in both untreated and treated ESCs, indicating a very low level of activation (data not shown). The addition of IGF-II at 500 ng/ml led to a strong phosphorylation of IGF-IR and InsR in untreated cells but not in treated cells, while LR3 IGF-I, an IGF-I analogue with a reduced affinity for IGFBPs, was able to activate the receptors in both untreated and treated cells, suggesting that the presence of IGFBPs in decidualized cells regulates the activation of receptors (Fig. 8A and B). The phosphorylation of IGF-IR and InsR by LR3 IGF-I was considerably more pronounced in decidualized cells in comparison with untreated cells, reflecting the higher level of functional protein expression in decidualized cells (Fig. 5C). In parallel, phosphorylated IRS-1 was not detected in the absence of added IGFs. As shown in Fig. 8C, IGF-II and LR3 IGF-I induced a similar level of IRS-1 phosphorylation in untreated cells. However, in decidualized cells, IRS-1 phosphorylation was stronger after LR3 IGF-I than after IGF-II treatment. In contrast, we observed phosphorylated IRS-2 in decidualized cells without the addition of IGFs and found that the phosphorylation increased at the same level in the presence of either IGF-II or LR3 IGF-I. This result indicates that IRS-2 could be activated despite high levels of IGFBPs in treated cells (Fig. 8D). Furthermore, tyrosine phosphorylated IRS-2 migrated significantly faster in decidualized than in non-decidualized cell extracts, in agreement with the total IRS-2 immunoblot (Fig. 7C).

Discussion

Differentiation of ESCs constitutes a crucial event during the preparation of the endometrium for blastocyst implantation and adequate placental development. Decidualization plays two apparently paradoxical roles in implantation. On the one hand, the trophoblast–decidual cell paracrine interactions are essential for placentation. On the other hand, decidual cells limit the depth of implantation, preventing the excessive trophoblast invasion observed in the absence of decidua, such as in tubal pregnancy or in the placenta percreta associated with scarring tissue (Goffin *et al.*, 2003b).

The present study demonstrates a strong modulation of IGF system component expression in human ESCs undergoing decidualization. Decidualizing treatment induced a significant up-regulation of IGF-II levels but not those of IGF-I. In the endometrium, IGF expression is well-known to be controlled by steroid hormones during the menstrual cycle. IGF-I has been described as a major 'oestromedin', reaching maximal expression during the late proliferative phase. In contrast, IGF-II is mainly expressed during the mid to late secretory phase, under the influence of progesterone (Zhou *et al.*, 1994; Gao *et al.*, 1999). Together, these observations argue for a role for IGF-II rather than for IGF-I in the decidualization process.

The activity of IGFs is strongly modulated by IGFBPs, since they control the availability of IGFs to receptors. IGFBP-1 is a major protein produced by the decidualized endometrium and was used in this study as a marker for ESC differentiation. Besides the high induction of IGFBP-1 levels, which could inhibit IGF action, we found increased IGFBP-4 levels in decidualized cells. Interestingly, IGFBP-4 immunoblots of ESC CM showed pronounced proteolysis of the



B

	0h	8h	1d	3d	6d	12d
IRS-1 mRNA fold change	1	1.80+/-0.29*	1.69+/-0.31*	2.33+/-0.63*	2.14+/-0.48*	1.42+/-0.48
IRS-2 mRNA fold change	1	1.67+/-0.48	1.37+/-0.15*	2.10+/-0.76*	1.77+/-0.34*	1.50+/-0.39

Figure 7 Expression time course of IRS-1 and IRS-2 during decidualization. (**A**) IRS-1 and IRS-2 mRNA levels measured by RT–PCR in ESC cultures. Total RNA was extracted from non-decidualized (ND) and decidualized (D) cells after 0, 8 h, 1, 3, 6 and 12 days of treatment. The presented results are from a single representative experiment. The densitometric values obtained for IRS-1 and IRS-2 mRNA at each time point were referred to the densitometric value at time 0 h considered as being equal to 1. (**B**) Fold induction of IRS-1 and IRS-2 mRNA levels. Table shows the ratio of densitometric values at each time point in decidualized versus non-decidualized cells. Data represent the mean and SD summarized from three independent experiments performed in duplicate. Statistical significance of the differences above value for time point 0 h was determined by the Student's *t*-test (*$P < 0.05$). (**C**) IRS-1 and IRS-2 protein levels measured by western blot. Immunoblotting of IRS-1 and IRS-2 was performed on protein extracts from untreated cells (−) and from cells treated with cAMP and MPA (+). Whole cell lysates were extracted after 0, 8 h, 1, 3, 6 and 12 days of culture. Experiments were repeated three times with similar results.

binding protein in treated as well as in untreated cells. *In vivo*, IGFBP-4 is the second most abundant IGFBP produced by the human decidua, after IGFBP-1 (Han and Carter, 2000). IGFBP-4 proteolysis generates fragments with highly reduced affinity for the IGFs and thus, represents a major process in the regulation of IGF bioavailability. We confirmed by *in vivo* immunoneutralization experiments that PAPP-A was the protease responsible for IGFBP-4 degradation. PAPP-A has been described as an IGF-II-dependent protease (Lawrence *et al*., 1999). Qin *et al*. (2000) have established that physical interaction between IGF-II and IGFBP-4 is required for the optimal proteolytic activity of PAPP-A. In the study of Giudice *et al*., (2002), the proteolysis of IGFBP-4 was examined by testing *in-vitro* the proteolytic activity of ESC conditioned medium on recombinant [^{125}I]IGFBP-4. In this case, [^{125}I]IGFBP-4 was only proteolyzed when exogenous IGF-II (5 nM ~40 ng/ml) was added. We have to emphasize that we performed our experiments in the absence of any exogenous IGF-II. It is possible that *in-vitro* studies do not reflect the real proteolytic activity of PAPP-A due to the presence of IGF-II in ESC medium. Moreover, it has been suggested in several papers that PAPP-A activity is not strictly IGF-dependent (Byun *et al*., 2001; Monget *et al*., 2003). Thus, we propose that either some fraction of IGF-II produced by the cells is able to activate PAPP-A and to induce proteolysis of endogenous IGFBP-4 or that cleavage of IGFBP-4 is due to a basal, IGF-independent activity of PAPP-A.

IGFBP-5 expression level was also affected by decidualizing treatment. We observed a strong decrease in mRNA levels, which became undetectable after 6 days of treatment. The mechanism of *IGFBP-5* gene repression in cAMP and progesterone-treated ESCs remains to be elucidated. It is of particular interest to note that prolactin is a strong inhibitor of IGFBP-5 expression in lactating mammary glands (Tonner *et al*., 1997). Since decidualizing cells produce large amounts of prolactin, this might explain our observation.

The actions of IGFs are mediated by IGF-IR and InsR. Both receptors levels increased during the period of treatment, with a higher induction observed for InsR. Furthermore, we report varying kinetics between the two InsR isoforms mRNA. The InsR-A mRNA level showed a stronger increase in decidualized cells than did the InsR-B mRNA level and InsR-A became the major isoform after 3 days of treatment. This is of particular interest since InsR-A presents a high affinity for IGF-II, in an order of magnitude comparable to IGF-IR. Their interaction has been implicated in fetal growth and the physiology of various cancers (Frasca *et al*., 1999). Thus, elevated levels of both IGF-IR and InsR-A in decidualized cells provide a higher binding capacity for locally available IGF-II.

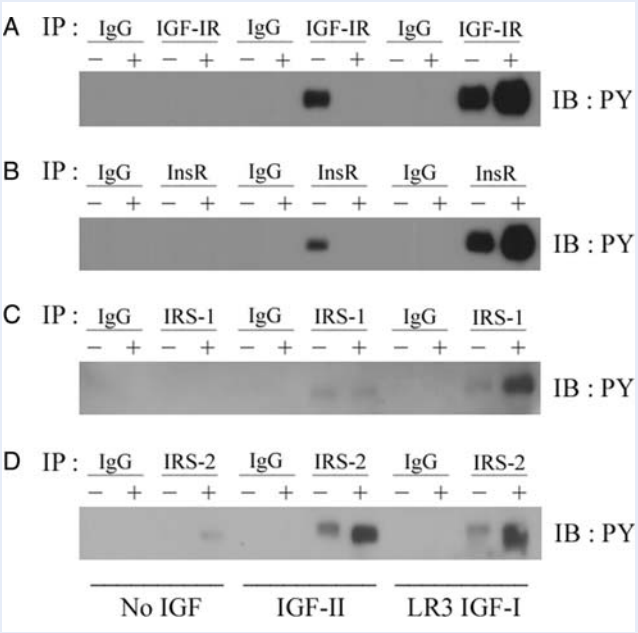


Figure 8 Phosphorylation status of IGF-IR, InsR, IRS-1 and IRS-2. Confluent ESCs were cultured in DMEM/F12 2% charcoal-treated FBS with 0.5 mM 8-br-cAMP and 1 μM MPA (+) or ethanol (−) for 6 days. IGF-II or Long R3 IGF-I (500 ng/ml) was added, or not, 15 min before protein extraction. Protein samples were immunoprecipitated with either anti-IGF-IR antibody (**A**), anti-InsR antibody (**B**), anti-IRS-1 antibody (**C**) or anti-IRS-2 antibody (**D**) in parallel with IgG control and then subjected to western blot with anti-phosphotyrosine 4G10 (PY) antibody. Experiments were repeated three times with similar results. IP, immunoprecipitation; IB, immunoblot.

We observed a higher expression of IRS-1 and IRS-2 in decidualized cells. Changes in docking protein expression occurred early, as observed in IGF-II, IGFBPs and receptors expression. This suggested involvement of the network in the decidualization process. To test this hypothesis, we investigated the phosphorylation status of receptors and docking proteins during ESC differentiation. Our results demonstrated that IGF-II produced by decidualized cells did not give rise to a sustained autophosphorylation of IGF-IR and InsR. We also tested the possibility that the two receptors could be activated in decidualized cells by a paracrine mechanism, since large amounts of IGF-II are produced by placental trophoblasts at the feto–maternal interface during placentation. The addition of exogenous IGF-II failed to phosphorylate IGF-IR and InsR in decidualized cells. This was probably due to the presence of IGFBPs, since treatment with LR3 IGF-I, presenting a reduced affinity for IGFBPs, induced high phosphorylation levels in both receptors. It is important to mention that immunoprecipitation with either IGF-IR or InsR-specific antibody can also lead to the recovery of hybrid IGF-I/Ins receptors. It is very likely that hybrid receptors are present in ESCs since they have been reported in most cell types expressing significant levels of both IGF-IR and InsR (Bailyes *et al.*, 1997; Federici *et al.*, 1997). So, the phosphotyrosine signals detected in immunoprecipitated extracts could correspond to the combination of phosphorylated homodimers and hybrid receptors. However, the exact contribution of hybrid receptors in the biological action of the IGFs is unclear. And understanding their exact role is rendered more difficult by the fact that InsR exists in two isoforms and that the relative abundance of the isoforms can lead to different effects in terms of ligand binding and signal transduction (Pandini *et al.*, 2002). Whether a higher proportion of hybrid receptors containing InsR-A than hybrid receptors containing InsR-B is present in decidualized ESCs remains to be elucidated, as does the way in which these hybrid receptors contribute to IGF signalling activity.

We observed a distinct pattern of IRS-1 and IRS-2 phosphorylation in basal and IGF-stimulated conditions. The IRS-1 pattern of activation was similar to that of the receptors. Indeed, in decidualized cells, we could detect a sustained IRS-1 tyrosine phosphorylation only after the addition of LR3 IGF-I to the culture medium. In contrast, IRS-2 was slightly phosphorylated in decidualized cells without the addition of IGFs and phosphorylation was significantly increased by the addition of IGF-II or LR3 IGF-I. It has been recently reported that a specific and sustained activation of IRS-2 was induced by IGFs through InsR in spite of low phosphorylation of the receptor (Denley *et al.*, 2007). Some studies had already suggested that high levels of InsR and IGF-IR phosphorylation might not be required for all biological actions of the receptors (Sbraccia *et al.*, 1990; Rafaeloff *et al.*, 1991; Blakesley *et al.*, 1995; Stannard *et al.*, 1995). Despite structural and functional similarities, IRS-1 and IRS-2 differ in some respects as proven by the study of targeted disruptions of the genes coding for the two adaptors (Withers *et al.*, 1999). Both proteins contain a specific set of multiple tyrosine phosphorylation sites at their C-terminus, allowing the recruitment of various SH2 domain-containing signalling molecules (White, 2002). In addition, the two IRS proteins might differ in their mode of interaction with InsR and IGF-IR (He *et al.*, 1996; Sawka-Verhelle *et al.*, 1996), suggesting distinct mechanisms of regulation. It would be interesting to find out which signalling proteins are recruited by IRS-2 activation in decidualized cells and which receptor is responsible for the adaptor activation. Furthermore, we observed a faster migrating IRS-2 in decidualized cells, which could be due to a lower serine phosphorylation level. Several studies have suggested that high serine/threonine phosphorylation of IRS is inhibitory for insulin signalling, since it has been shown to reduce tyrosine phosphorylation (Tanti *et al.*, 1994; Eldar-Finkelman and Krebs, 1997; Sharfi and Eldar-Finkelman, 2008). Therefore, specific regulation of IRS-2 phosphorylation could occur in decidualized cells, favouring signalling activation.

In conclusion, our overall results suggest that the IGF signalling pathway is only weakly activated by autocrine IGF-II during *in-vitro* differentiation of ESCs. But the profound changes in the expression of its components indicate that the signalling pathway is ready to be activated. We propose that *in vivo*, once placentation occurs and a high level of IGF-II is present at the feto–maternal interface, the IGF signalling pathway could be fully activated to act in the maintenance and in the growth of decidualized endometrial cells, mediated mostly by IRS-2 activation. It has been shown that IGFs can influence the production of IGFBP-1 and PRL and stimulate the growth of ESCs (Frost *et al.*, 1993; Irwin *et al.*, 1993, 1994; Tang *et al.*, 1994; Tseng and Mazella, 2002). High level expression of endometrial IGFBPs and trophoblatic IGF-II and IGFBP proteases could thus allow a localized and a strongly controlled action of IGF-II on the trophicity of the basilar decidua during pregnancy.

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