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Insulin-like growth factor 2 and short-range stimulatory loops in control of human placental growth

Rolf Ohlsson[1], Lars Holmgren[1], Anna Glaser[1], Agnieszka Szpecht[1,2] and Susan Pfeifer-Ohlsson[1,3]

[1]Centre for Biotechnology, Karolinska Institute, Huddinge University Hospital F82, S-141 86 Huddinge, Sweden, [2]Department of Genetics, University of Warsaw, Al. Ujadowskie 4, 00-478 Warsaw, Poland and [3]Department of Pediatrics, Huddinge University Hospital, S-141-86 Huddinge, Sweden

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Substructures of the first-trimester human placenta (within 3 months post-conception) display 'pseudomalignant' properties. We show here, by *in situ* hybridization, that the insulin-like growth factor 2 (IGF-2) gene expression is particularly active in the cytotrophoblasts, which dominate these structures. Because the majority of placental IGF-2 mRNA is polysomal in extracts of first-trimester placenta, the spatial pattern of IGF-2 transcripts generally also defines the pattern of IGF-2 production. In primary trophoblast cultures, rendered quiescent by serum starvation, IGF-2 performs as a human embryonic growth factor by activating cell cycle entry/progression. Although both type 1 and 2 IGF receptor mRNAs can be found co-distributed with IGF-2 mRNA during placental development (supporting an autocrine role for IGF-2), these occasional patterns are confined to cytotrophoblasts with low proliferative potential. The reciprocity in ligand and receptor expression patterns are discussed in terms of rate-limiting steps in the involvement of IGF-2 in the proliferative phenotype of the early human placenta.

Key words: human placenta/first-trimester/IGF-2/cytotrophoblasts

Introduction

The human placenta undergoes dramatic structural reorganizations during pregnancy in order to synchronize functionally with the development of the embryonic/fetal and maternal tissue compartments. The spatio-temporal patterns of these events will eventually establish a vectorial nutrient exchange function between embryonic/fetal and maternal blood circulatory systems. In early pregnancy (the first 3 months following conception), the trophoblast lineage(s) dominates the placental phenotype, due to properties such as explosive growth and invasiveness. In conjuction with the immune privilege of the trophoblasts (which are facing the circulating maternal blood), these 'pseudomalignant' properties of the trophoblasts are reminiscent of some aspects of cancerous transformation, where normal regulatory growth controls have been short-circuited (Ohlsson and Pfeifer-Ohlsson, 1987; Ohlsson *et al.*, 1989a).

Since the placental architecture undergoes continuous remodeling throughout pregnancy, the cytotrophoblast populations are integrated in different substructures at different developmental stages of human pregnancy. Pertinent to this report, the most proliferative cytotrophoblasts form a continuous sheet (or shell) which will very rapidly expand during the first month following blastocyst implantation. This provides space for development of the embryo proper, as well as establishing the future placental bed (Langman, 1969; Hamilton and Hamilton, 1977; Ohlsson *et al.*, 1989). Later in first-trimester pregnancies, the proliferation of trophoblasts in shell structures is retarded while other cytotrophoblasts will, in columnar protrusions of chorionic villi, for example, display a highly proliferative phenotype persistent through the major part of human placental development (Langman, 1969; Hamilton and Hamilton, 1977; Ohlsson *et al.*, 1989).

Because circulatory blood systems are poorly developed in the placentae during very early stages of development (Langman, 1969; Hamilton and Hamilton, 1977), short-range stimulatory loops in control of trophoblast growth have been invoked to explain the rapid expansion of the trophoblast cell component. For example, locally produced PDGF or PDGF-like activities have been proposed to contribute to the expansion of the trophoblast cell component of early pregnancy; potentially through autocrine/short-range paracrine loop formations (Goustin *et al.*, 1985; Ohlsson and Pfeifer-Ohlsson, 1986; L.Holmgren *et al.*, manuscript in preparation). Because activation of the trophoblast insulin-like growth factor 2 (IGF-2) gene is a post-implantation event that correlates with the formation of the highly proliferative trophoblastic shell (R.Ohlsson *et al.*, 1989b), IGF-2 may be yet another member in a set of gene products that orchestrate early human placenta development.

IGF-2, a close relative to IGF-1 and insulin (Dafgård *et al.*, 1985), has been implicated as a growth-promoting factor preferential for mammalian pre-adolescent development (Scott *et al.*, 1985; Schofield and Tate, 1987; Engström and Heath, 1988; Gray *et al.*, 1987). The pathways through which IGF-2 might function in embryonic micro-environments are unclear, however, since IGF-2 displays high affinities to both IGF-1 and IGF-2 receptors (Czech *et al.*, 1984). It is generally believed that the IGF-1 receptor mediates IGF-2-triggered post-receptor pathways that lead to cell proliferation (Czech *et al.*, 1985), although it has been proposed that the IGF-2 receptor may mediate a growth factor-triggered cytoplasmic signal (Tally *et al.*, 1987). Therefore, both target cell identities and functional pathways *in vivo* have been poorly resolved for IGF-2.

We report here that the IGF-2 gene, like the PDGF-B chain gene (Goustin *et al.*, 1985), is expressed at very high levels in cytotrophoblasts and also in mesenchymal stroma cells, to a lesser degree. Because IGF-2 induces cell cycle entry and progression of quiescent primary cultures enriched in either trophoblasts or mesenchymal stroma cells, IGF-2

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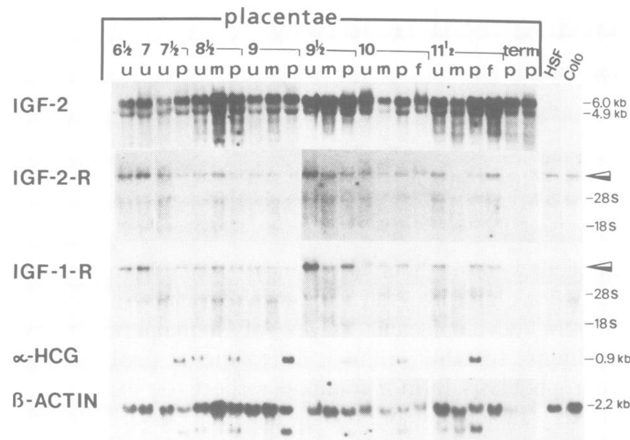


Fig. 1. Expression of IGF-2 gene during human development. Ten μg/lane of total RNA, extracted from staged human aborted tissues were subjected to electrophoresis and blot analysis using ^{32}P-labeled specific hybridization probes as detailed in the figure and Materials and methods (u, aliquot of unfractionated abortus; m, membranes enriched in endometrial tissue; p, pure placental chorionic villi; f, fetus). The chorionic gonadotropin α-subunit (α-HCG) and β-actin mRNA levels were used as diagnostic markers for the fractionation procedure and input mRNA respectively. The positions of type 1 and 2 IGF receptors are depicted by arrows in the Figure.

is perceived as an important modulator of placental cell proliferation and maturation. The trophoblast target cells *in vivo* for IGF-2 were defined by analyzing the spatial distribution of IGF-1 and IGF-2 receptors by *in situ* hybridization to thin sections of formalin-fixed first-trimester placentae. The transient formations of IGF-2 stimulatory loops that participate in the control of human placental development are discussed.

Results

Expression of the IGF-2 gene and its receptors during placental development

The expression of the IGF-2 gene was analyzed in relation to both extra-embryonic/embryonic compartments and to different stages of placental development. In particular, the aim was to establish the pattern of expression of IGF-2 and its receptors by fractionating the aborted tissue material from several gestational stages into unfractionated, membranes, pure placental chorionic structures and, occasionally, embryo proper. The purity of the placenta chorionic fraction, as well as total mRNA input, was assessed by analyzing the α-subunit of human chorionic gonadotropin (HCG) and β-actin mRNA levels respectively (Figure 1). Successive Northern blot analysis of the extracted RNA revealed that neither the type 1 and 2 IGF receptor nor the IGF-2 gene expression patterns displayed any marked developmental profile (Figure 1). The HCG α-subunit mRNA levels confirmed the enrichment of placenta chorion from the mixed embryonic material. The expression of IGF-2 in maternal decidua, enriched in the m fraction in Figure 1 (more specifically in spiral arteries; R.Ohlsson and H.Luthman, in preparation), was unexpected in view of the role of IGF-2 as an embryonic/fetal growth factor.

Spatial distribution of IGF-2 transcripts during placental development

Given the dramatic changes in cell type composition during placental development of first-trimester pregnancies, it was conceivable that only a subset of extra-embryonic cells at any developmental stage harbored active genes for IGF-2 and its receptors. To examine this issue in detail we employed *in situ* hybridization analysis of thin sections of formalin-fixed, paraffin-embedded placental tissue. Figure 2 shows that a ^{35}S-labeled antisense IGF-2 riboprobe localizes highly active IGF-2 genes, primarily to the highly proliferative cytotrophoblastic columns that protrude from a chorionic villus of a first trimester placenta (panels a–e; the essential structural details of the first-trimester placenta and their proliferative properties are indicated in panel a and in the legend of Figure 2, to facilitate interpretation). In addition, IGF-2 transcripts can be detected in both villous cytotrophoblasts and mesenchymal stroma cells, albeit at much reduced levels in comparison with the very proliferative trophoblasts (trophoblastic shell or columnar trophoblasts; panel a–c and data not shown; compare IGF-2 gene expression in mesenchyme of term placenta in Figure 2, panel f). Term cytotrophoblast layers also express the IGF-2 gene very actively (data not shown). With the demonstration that the bulk of IGF-2 transcripts are engaged in translation processes (see below), we conclude that the spatial distribution of IGF-2 transcripts also define the site of IGF-2 production.

Regulation of IGF-2 expression during trophoblast proliferation and differentiation

It is noteworthy that we have not in any instance found IGF-2 transcripts in the differentiated, non-proliferative syncytiotrophoblastic cell layers, suggesting that IGF-2 gene expression is permanently down-regulated in association with the trophoblast differentiation process. To examine this issue in some detail we used the *in vitro* differentiation model of normal trophoblasts purified from term placentae (Kliman *et al.*, 1986). Such cells express high levels of IGF-2 mRNA *in vivo* (data not shown) and undergo *in vitro* morphological differentiation to form syncytia in the presence of 20% fetal calf serum within 4 days (Figure 3A). In Figure 3B it is shown that in purified term trophoblasts undergoing spontaneous differentiation (initiated within 2 days of *in vitro* incubation), the levels of the 6.0, 4.9 and 2.2 kb IGF-2 mRNAs were initially very high, but synchronously down-regulated (>20-fold) prior to syncytia formation. In contrast, the β-actin mRNA levels remained relatively constant. Although this result could be anticipated from the *in situ* hybridization studies, it was surprising to learn that the transcriptional activity of the IGF-2 gene was not appreciably modulated during *in vitro* trophoblast differentiation (within a 4-day period), as analyzed by nuclear run-on transcription assays (Figure 3C). Moreover, neither the IGF-1 nor the β-actin gene rates of transcription were substantially modulated in the same cells (Figure 3C). We therefore conclude that IGF-2 expression is regulated at the post-transcriptional level during trophoblast differentiation *in vitro*.

To investigate potential negative controls of IGF-2 expression at the translational level, we analyzed polysomal

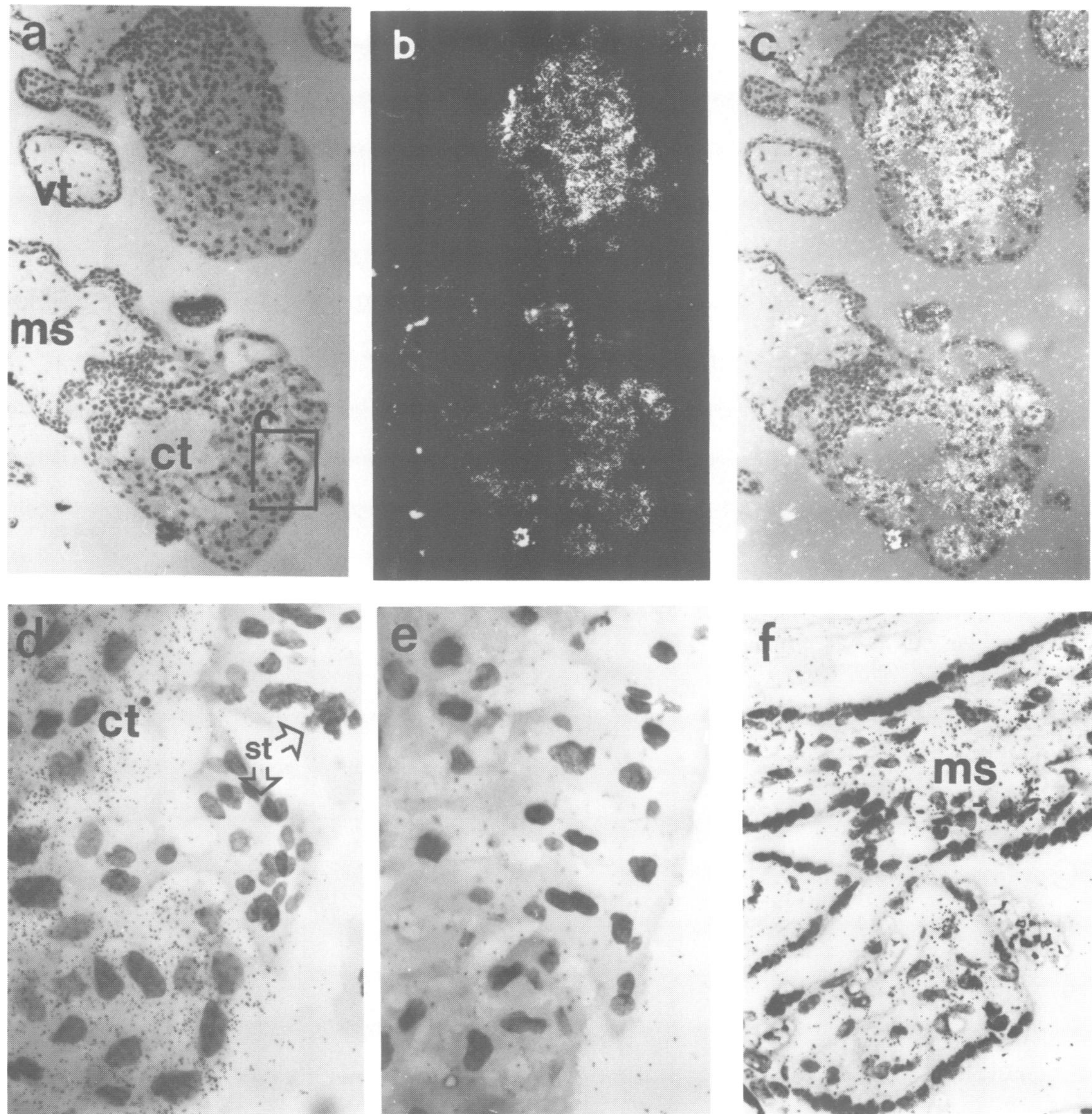
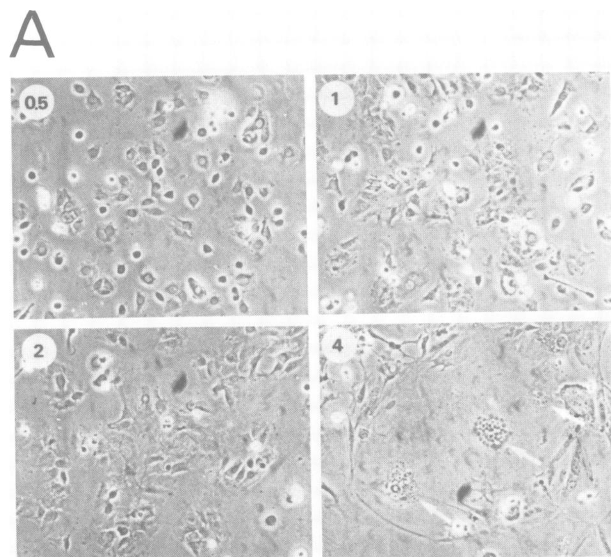


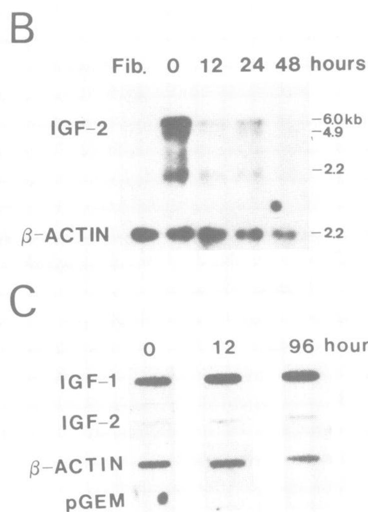
Fig. 2. Spatial distribution of IGF-2 transcripts in human placentae of first- and third-trimester pregnancies. (**a–c**) Bright-field, dark-field and composite views respectively of IGF-2 gene expression in a 5-week human placenta. (**d**) Enlargement to show silver grain distribution over only columnar cytotrophoblasts (ct). Note the absence of silver grains over the syncytiotrophoblasts (st, arrows) in panel d and villous trophoblast (vt) and mesenchymal stroma (ms) in panel a. (**e**) Control hybridization of adjacent section with sense ^{35}S-labeled IGF-2 riboprobe. (**f**) IGF-2 gene expression in term placenta is generally confined to the mesenchymal stroma (ms). The relative proliferative potential of the various placental structures in first-trimester placenta is: trophoblastic shell/columnar trophoblast > villous cytotrophoblasts > mesenchymal stroma > > syncytiotrophoblasts (Pfeifer-Ohlsson *et al.*, 1984; Ohlsson *et al.*, 1989). Magnifications are 86-fold (a–c), 399-fold (d) and 172-fold (e).

IGF-2 transcripts in the cytoplasm of first-trimester placentae. Figure 4A shows a sucrose gradient fractionation of polysomes extracted from a first-trimester human placenta. Following RNA isolation and slot-blot hybridization analysis of individual fractions, it was evident that the majority of IGF-2 mRNA is polysomal and thus engaged in the translation process. Controls were provided by EDTA-treated extracts with disrupted polysomal structure. In Figure 4B it is shown that the bulk of IGF-2 transcripts in such samples distribute with mRNA that is not engaged in the translational process. We therefore conclude that there is little, if any, control of IGF-2 expression at the translational level in developing human placenta.

IGF-2 as a placental growth factor

To establish a growth potential, purified IGF-2 was added to density-arrested and serum-starved placental cell cultures, enriched in either mesenchymal stroma cells or cytotrophoblasts. Figure 5A shows primary cultures of first-trimester trophoblasts and fibroblasts, which were used in a cell-cycle assay. These cells were rendered quiescent by serum starvation for 3 days prior to addition of IGF-2. Following pulse-labeling for 2 h with [^{3}H-methyl]thymidine at the end of each time point, ethanol-fixation and autoradiography, we could conclude that between 6 and 11% of both cell types, reached the S phase following 18–20 hours exposure to 20 ng/ml of IGF-2 (Figure 5B). This result





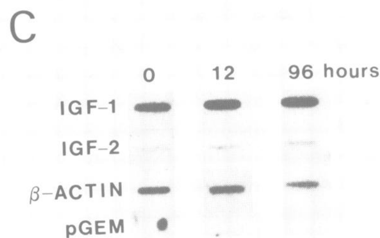


Fig. 3. Regulation of IGF-2 expression in term trophoblasts, spontaneously undergoing *in vitro* differentiation. Purified term trophoblasts adhere to substratum within 0.5 days and differentiate into syncytium (arrows) within 4 days as shown in **panel A** (77-fold magnification). Northern blot analysis of RNA (~5 μg/lane) extracted from trophoblasts undergoing *in vitro* differentiation (**B**). The IGF-2 mRNA levels during *in vitro* differentiation process were related to β-actin mRNA levels. The fib. lane shows level of IGF-2 mRNA in term placenta fibroblast primary cultures (passage 5) (**B**). Run-on transcriptional analysis of trophoblastic nuclei, isolated from a differentiation time course (**C**).

might reflect a modest low growth stimulatory effect by IGF-2 *per se*. This deduction is certainly in line with the fact that the IGF-2 mRNA abundancy is one or two magnitudes higher than that of c-*sis* mRNA, for example, which encodes a considerably more potent mitogen.

Alternatively, IGF-2 functions as a potent growth factor for a low proportion of cells with abundant receptor numbers, a deduction supported by the pattern of type 1 and 2 receptor expression in human placentae *in vivo* (see below). Nonetheless, these data establish that IGF-2 can participate in the formation/maintenance of the proliferative phenotype of human placentae.

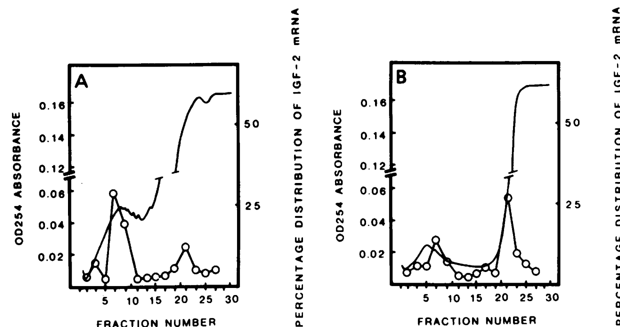
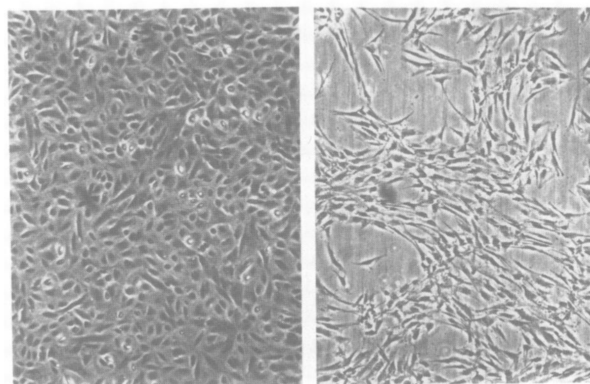


Fig. 4. Analysis of polysomal IGF-2 transcripts. Cytosolic extract of a first-trimester placenta was loaded on a sucrose gradient in the absence (**A**) or presence (**B**) of EDTA and subjected to high-speed centrifugation. Following fractionation of the gradients, total RNA was extracted from every other fraction and analyzed for presence of IGF-2 mRNA sequences by slot-blot hybridization. The levels of IGF-2 transcripts, presented in arbitrary units, has been derived from spectrographic measurement of X-ray film.



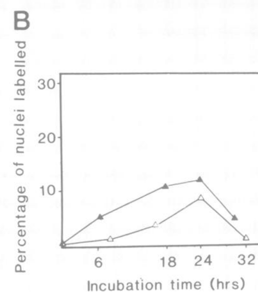


Fig. 5. IGF-2 as a placental growth factor. Primary cultures of trophoblasts and mesenchymal stroma cells (**A**), were starved for three days in MCDB 104 medium complemented with 1 mg/ml bovine serum albumin. Following addition of IGF-2 (20 ng/ml), cell proliferation was monitored by [^{3}H-methyl]thymidine incorporation for 2 h prior to formalin-fixation autoradiography (**B**). Data are presented as percentage S-phase nuclei that can be monitored during cell cycle traverse, triggered by IGF-2. Open and solid symbols show growth stimulation analysis using trophoblast and mesenchymal stroma primary cultures respectively.

Spatial overlap between IGF-2 and type 1 and 2 IGF-receptor gene expression

To establish formally the possibility for an involvement of endogenously produced IGF-2 in short-range stimulatory loops of growth control in human placenta *in vivo*, we

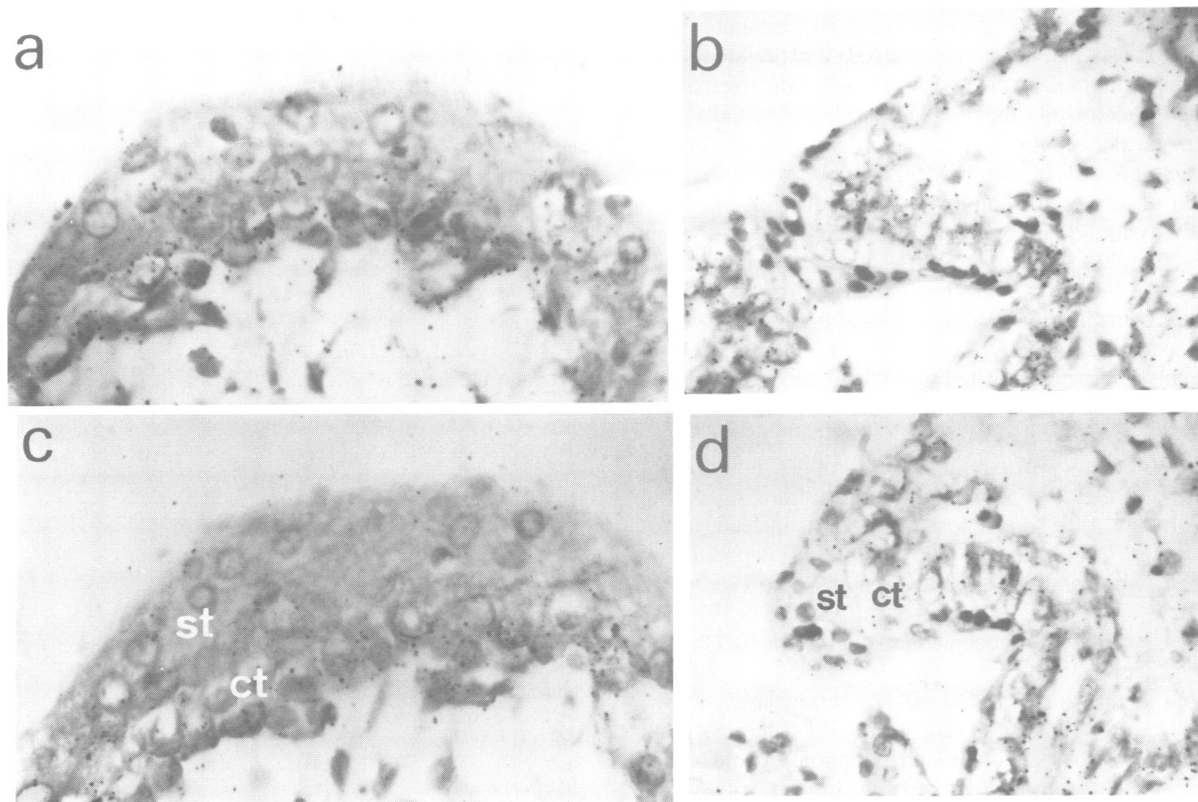


Fig. 6. Co-distribution of IGF-2 and type 1 and 2 IGF-receptor mRNAs in first trimester placenta. ^{35}S-labeled DNA probes of IGF-2 and IGF-1 receptor, as well as ^{32}P-labeled IGF-2 receptor oligonucleotide, were hybridized to serial thin sections of a 7-week-old human placenta. **Panels A** and **B** show the selective distribution of IGF-2 mRNA expression to cytotrophoblasts of chorionic villi in two separate motifs. The corresponding regions in adjacent thin sections also express IGF-1 receptor (**panel C**) and IGF-2 receptor mRNAs (**panel D**). The syncytiotrophoblast (st) and cytotrophoblast (ct) cell layers are indicated in the figure. Magnifications are 432-fold (A and C) and 371-fold (B and D).

analyzed the simultaneous presence of IGF-2 and type 1 and type 2 receptors by *in situ* hybridization analysis of adjacent thin sections of a formalin-fixed paraffin-embedded first-trimester placenta. Figure 6 shows the overlapping presence of IGF-2 and either type 1 or type 2 receptor in villous cytotrophoblasts (such cells display a relatively poor proliferative potential in contrast to cytotrophoblasts of the shell and columnar structures). The highest levels of type 1 and 2 receptor mRNAs were generally found in cytotrophoblasts of secondary villi (Figure 6), with occasional expression detected in more proliferative trophoblasts (which constitute columnar trophoblasts and trophoblastic shell, for example). It should be pointed out, however, that the expression of both receptor types was often at or beyond the limit of detection, as analyzed by *in situ* hybridization. This observation is particularly relevant for the trophoblastic shell and columnar cytotrophoblasts, both of which display the highest levels of IGF-2 transcripts while no receptor mRNAs could generally be detected (data not shown). In contrast, spiral arteries of maternal endometrial tissue, present in the same thin sections, expressed much higher levels of both types of receptor (R.Ohlsson and H.Luthman, in preparation). We therefore conclude that a reciprocity in ligand and receptor expression patterns influences IGF-2 short-range function(s), to be restricted at the receptor level in highly proliferative cytotrophoblasts.

Discussion

The down-regulation of the proliferative phenotype of the human placenta during the second and third trimester (Langman, 1969; Hamilton and Hamilton, 1977), is correlated with c-*myc* gene expression patterns (Pfeifer-Ohlsson *et al.*, 1984; Ohlsson and Pfeifer-Ohlsson, 1986). In contrast, the even expression pattern of mRNAs for either IGF-2 or type 1 and 2 IGF receptors did not display any apparent correlation with human placental growth patterns. The spatial distribution of IGF-2 transcripts revealed, however, that in early human placentae, the highest IGF-2 mRNA levels were confined to the most proliferative cell components of early human placenta (trophoblastic shell, columnar trophoblasts or trophoblastic islands), while the villous cytotrophoblasts and, throughout placental development, the mesenchymal stroma cells harbored less active IGF-2 genes. The close correlation between high IGF-2 expression and highly proliferative phenotypes in first-trimester pregnancies might, therefore, also reflect IGF-2 involvement in endogenous stimulatory pathways to promote cell proliferation. The spatial distribution of IGF-2 mRNA in term placenta is reminiscent of first-trimester pregnancies, since term cytotrophoblasts express much higher levels of IGF-2 mRNA than mesenchymal stroma cells, as shown by both *in situ* and Northern blot hybridization analysis of thin sections and extracted RNA from purified term placenta cell

components. In *in vitro* differentiation experiments, term cytotrophoblasts will down-regulate IGF-2 expression at the post-transcriptional level, prior to syncytia formation. Although this result may explain at least partially, the mechanism that regulates the *in vivo* distribution of IGF-2 transcripts in early human placenta, the poor proliferative potential of term cytotrophoblasts (Kliman *et al.*, 1986) is in conflict with IGF-2 expression levels. The highest levels of IGF-2 expression that we have detected in term placenta are confined to a trophoblastic shell structure, as assessed by *in situ* hybridization analysis. A role for IGF-2 in these incidences might involve cross-border communication. Although we have established that IGF-2 can perform as a growth factor for both cytotrophoblasts and mesenschymal cells of first trimester pregnancies, it is unlikely that the poor *in vitro* stimulatory effect of IGF-2 implies a dominant promotion to yield the very rapid cell proliferation during the second and fifth weeks post-conception. Instead, a role for IGF-2 in the expansion of trophoblast cell population during early placenta development may involve synergistic interactions with other placental growth factors, like PDGF-B and TGFβ1, to lower mitogenic threshold levels (R.Ohlsson *et al.*, in preparation).

Given that the bulk of IGF-2 mRNA is translated *in vivo*, the spatial co-distribution of IGF-2 mRNAs with type 1 and 2 receptor mRNAs should identify regions where endogenous stimulatory loops may operate. Indeed, mRNA for either type of receptor is co-localized with IGF-2 mRNA in villous cytotrophoblasts. The low hybridization signals for both receptor types in mesenchymal stroma and highly proliferative trophoblasts, however, suggest that IGF-2-induced rapid placental cell proliferation is restricted primarily by limitations in the receptor numbers. Likewise, PDGF-B-mediated growth of human trophoblasts through stimulatory loops appears to be restricted at the post-transcriptional level of receptor expression, to yield a low proportion of trophoblasts with co-expressed trophoblast PDGF-B ligand and PDGF-B receptors (Goustin *et al.*, 1985; L.Holmgren *et al.*, in preparation). Such events, to maintain a low proportion of cells with co-expressed growth factor and receptor genes, represent key developmental controls that should normally operate to avoid the unprogrammed cell proliferation of neoplasia (Ohlsson and Pfeifer-Ohlsson, 1987).

In conclusion, this report has shown that IGF-2 is expressed primarily in highly proliferative cytotrophoblast structures, which dominate the overall placenta structure during the first few months following conception. Because the type 1 receptor is transiently co-expressed with its ligand, IGF-2 is likely to promote both trophoblast and mesenchymal growth in early pregnancy, through autocrine and short-range paracrine fashions. However, the reciprocity in ligand and receptor gene expression identifies a potential negative feedback mechanism that restricts receptor expression in tissue structures with highest levels of IGF-2 expression.

Materials and methods

Placental cell cultures

Primary cultures of first-trimester placentae, obtained by routine elective abortion at the Huddinge University Hospital, were established essentially as has been described (Goustin *et al.*, 1985). Many attempts underlied the isolation of trophoblast primary cultures, as small amounts of contaminating mesenchymal stroma cells rapidly overgrew trophoblast primary cultures. Cold ethanol-fixed primary cultures of trophoblasts and mesenchymal stroma cells were characterized by immunoperoxidase staining (Vectastain) of vimentin and cytokeratin (pKK1 monoclonal antibody, Labsystems Oy, Helsinki). Following serum withdrawal for 3 days, purified IGF-2 (a kind gift of Dr R.Humbel), was added at indicated time points to cells maintained in MCDB104 medium (Gibco), supplemented with 1 mg/ml bovine serum albumin (BSA). To detect S phase nuclei, the cultures received a pulse of 50 μCi/ml [^{3}H-methyl]thymidine (Amersham) as specified in the legend of Figure 5. Following fixation in cold ethanol, the cells were subjected to autoradiography to monitor S phase entry. Term cytotrophoblasts were Percoll gradient-purified from Cesarian sections and maintained *in vitro* according to Kliman *et al.* (1986).

Analysis of polysomal IGF-2 mRNA

To assess translatability of IGF-2 mRNA, sucrose gradient fractionation analysis, 10–60% sucrose (w/v) SW40 rotor for 2.5 hours, of first-trimester placentae polysomes was performed in the absence or presence of EDTA as has been described (Meyuhas *et al.*, 1987). Total RNA was extracted from every other fraction and subjected to blot hybridization analysis (Pfeifer-Ohlsson *et al.*, 1984).

Hybridization analysis

The probes used in this study were: a 0.68 kb *Hin*fI–*Pst*I fragment, encompassing the coding region of human IGF-2 cDNA cloned in pGEM3 (R.J.Wilkins *et al.*, submitted); human IGF-1 receptor cDNA pIGF-I-R.85, a kind gift of Dr A.Ullrich (Ullrich *et al.*, 1986); IGF-1 cDNA clone (containing a 650 bp insert), a kind gift of Dr J.Scott; human IGF-2 receptor oligonucleotide (30 bases encompassing 15 bases upstream and downstream of IGF-2 receptor termination codon: GGCACTGCGGAGTCAGA-TGTGTAAGAGGTC (Morgan *et al.*, 1987); β-actin (PAL41 plasmid); human chorionic gonadotropin (HCG) α-subunit, a gift of Dr H.C.Fiddes. Electrophoresis and transfer of total embryonic RNA as well as subsequent hybridization analysis was performed as has been described (Pfeifer-Ohlsson *et al.*, 1984). The DNA probes were radioactively labeled as follows: nick translation (IGF-2 and IGF-1 receptor), SP6/T7 RNA riboprobes (IGF-2; Promega) or 3′-end labeling of oligonucleotide (IGF-2 receptor) by a terminal transferase kit (IBI) in the presence of [^{32}P]dATP (Amersham). *In situ* hybridization analysis of IGF-2 expression was performed for 2 h at 52°C on pretreated 4 μm paraffin sections of formalin-fixed human placentae of first- and third-trimester pregnancies (Pfeifer-Ohlsson *et al.*, 1984, 1985; Goustin *et al.*, 1985), using ^{35}S-labeled sense and anti-sense IGF-2 riboprobes. Analysis of co-distribution of IGF-2 and type 1 and 2 IGF receptors was performed for 30 h at 37°C by using nick-translated [^{35}S]DNA probes in the presence of 100 mM dithiothreitol, essentially as described (Pfeifer-Ohlsson *et al.*, 1984, 1985; Goustin *et al.*, 1985). Cytohybridization analysis of ^{32}P-labeled IGF-2 receptor was performed for 30 h at 37°C. Following application of Ilford K5 emulsion and exposure for 2-8 days, the sections were developed and counterstained as has been described (Pfeifer-Ohlsson *et al.*, 1984).

Nuclear run-on transcription analysis

The assessment of transcriptional rate of IGF-2 and control genes was performed by a run-on assay on detergent-prepared trophoblast nuclei that were obtained during an *in vitro* differentiation time course (Greenberg and Ziff, 1984; Smeland *et al.*, 1987). The ^{32}P-labeled nuclear RNA was purified by extensive DNase 1 treatment (RQ1, Promega), followed by Sephadex gel filtration and phenol extraction. Hybridization to slot blot filters, containing 5 μg of $CsCl_2$ gradient-purified plasmid DNA, followed previously published procedures (Greenberg and Ziff, 1984; Smeland *et al.*, 1987).

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