IGF-I and IGF-II mRNA expression in primary cultures of fetal hepatocytes

Expresión de IGF-I y II en cultivos primarios de hepatocitos fetales

GOYA, L.; DE LA PUENTE, A.; RAMOS, S.; ÁLVAREZ, C. and PASCUAL-LEONE, A. M.
Instituto de Bioquímica (Centro Mixto CSIC-UCM). Facultad de Farmacia. Ciudad Universitaria. 28040 Madrid. (Spain). Phone/Fax: 34 1 5438649

ABSTRACT

Secretion of IGFs in adult animals is mainly regulated by growth hormone (GH), but the role of other factors becomes principal during the perinatal period of the rat, when the IGF response to GH is not yet fully established. *In vitro* models overcome the simultaneous fluctuations of fuels and hormones that occur in live animals and are useful to investigate the underlying mechanisms by which hormones and nutrients regulate IGF secretion. A selective primary culture of fetal rat hepatocytes has been established in our laboratory in order to elucidate the molecular mechanism of action of different factors and conditions on IGF-I and -II gene expression during the perinatal period of the rat. In this preliminary study we report the regulatory role of serum on IGF-I and -II gene expression during stages of perinatal development. This system should be a useful tool for further studies of molecular mechanisms of IGF-I and -II regulation.

Key words: IGFs regulation. Serum factors. Fetal hepatocyte culture.

RESUMEN

La secreción de IGFs en animales adultos esta regulada principalmente por la hormona del crecimiento (GH), pero el papel de otros factores se convierte en principal durante el periodo perinatal de la rata, cuando la respuesta de la IGF a la GH aún no se ha estblecido completamente. Los modelos *in vitro* pasan por alto las fluctuaciones simultáneas de combustibles y hormonas que ocurren en el animal vivo y son útiles para investigar los mecanismos subyacentes por los que hormonas y nutrientes regulan la secreción de IGF. En nuestro laboratorio hemos establecido un cultivo primario selectivo de hepatocitos de feto de rata con el fin de aclarar los mecanismos moleculares de la acción de diferentes factores y condiciones sobre la expresión génica de IGF-I e IGF-II durante el periodo perinatal de la rata. En este estudio preliminar presentamos el papel regulador del suero sobre la expresión génica de IGF-I e IGF-II durante distintos estados de desarrollo perinatal. Este sistema puede ser una herramienta útil para posteriores estudios sobre los mecanismos moleculares de la regulación de IGF-I e IGF-II.

Palabras clave: Regulación de IGFs. Factores séricos. Cultivo de hepatocitos fetales.

Recibido: 12-12-96.
Aceptado: 20-12-96.
BIBLID [0004-2927(1996) 37:4; 707-717]

INTRODUCTION

Insulin-like growth factors IGF-I and -II (IGFs) are peptides that are structurally related to pro-insulin (1). IGFs are cell growth regulators that originate largely in the liver. Hepatic production of IGFs appears to be regulated at pretranslational levels, as indicated by the strong correlations between circulating IGFs and abundance of hepatic IGF-I and -II mRNA (2,3,4). Secretion of both IGFs and IGFBPs in adult animals is mainly regulated by growth hormone (GH), but nutritional status and serum insulin concentration are important factors involved in the regulation of IGFs synthesis and secretion (1). Their role as regulatory factors becomes principal during the perinatal period of the rat, when the IGF response to GH is not yet well established. We have recently demonstrated that refeeding and insulin treatment of undernourished and diabetic neonatal rats respectively lead to a recovery of serum and liver mRNA expression of IGF-I and -II without a prior increase of serum GH (3). These results strongly suggest that during the rat's perinatal period IGFs regulation is GH-independent. Therefore, it seemed appropiate to perform further experiments examining other putative regulatory factors, such as glucose and serum factors.

In experiments *in vivo* the simultaneous fluctuations of fuels and hormones that occur in diabetic and undernourished animals make it difficult to demonstrate specific regulation, thus, an *in vitro* model is required to investigate the underlying mechanisms. The *in vitro* system that more closely resembles normal developing liver is the primary culture of fetal hepatocytes. Since IGFs expression in primary cultures is limited and subject to plastic substratum-induced changes in the differentiation state of the liver cells, very scant data is available in the literature about the response of fetal hepatocytes in culture to different conditions. To overcome these difficulties, a selective primary culture of fetal hepatocytes has been established in our laboratory in order to unequivocally show the specific effect of different factors and conditions on IGF-I and -II gene expression during the mammalian perinatal period as well as to elucidate their molecular mechanism of action. In this preliminary study we report the role of serum, glucose and GH on IGF-I and -II gene expression during stages of perinatal development. The results also demonstrate that fetal rat hepatocytes retain some of the characteristics of the rat fetal liver while maintained in short term culture. This system should be a useful tool for further studies of molecular mechanisms of IGF-I and -II regulation.

MATERIALS AND METHODS

Materials

Recombinant human IGF-I and II (Boehringer Mannheim, Leverkusen, Germany) were used as standard and for iodination. RNAse A and RNAse T1 were also purchased from Boehringer Mannheim. Norditropin 4UI (growth hormone) was purchased from Novo Nordisk Pharma, S.A. Madrid (Spain). Fetal bovine serum was purchased from Biowhittaker (Ingelheim Diagnóstica y tecnología, S.A. Barcelona (Spain). Hyperfilm-MP autoradiography film were obtained from Amersham (Amersham Ibérica, S.A., Madrid, Spain). (32P)UTP was purchased from ICN (Nuclear Ibérica, S.A. Madrid, Spain). Riboprobe Gemini II Core System (Promega Corporation, Madison, WI, USA) was used for the generation of RNA probes. Bovine serum albumin and all other reagents were purchased from Sigma Chem. Co. (St. Louis, MO, USA).

Experimental models

Wistar rats bred in our laboratory with controlled temperature and artificial dark-light cycle were used throughout the study. Females were caged with males and mating was confirmed by the presence of spermatozoa in a vaginal smear. Each dam was housed individually from the 14th day of pregnancy. Animals were fed a standard laboratory diet ad libitum. Dams were killed and fetuses were exposed after abdominal incision. All experiments were conducted in accordance with the principles and procedures outlined in the NIH guide for care and use of experimental animals (Bethesda, MD, USA).

Cell extraction

Primary cultures of hepatocytes from 21 day-old Wistar rat fetuses were prepared by a non-perfusion collagenase dispersion method (5) that involves incubation of the minced tissue with Ca2+-free Krebs bicarbonate buffer containing 0.5 mM EGTA in a 150 ml conical flask for 30 min at 37 °C in a shaking water bath (100 cycles/min) under continuous gassing (O2/CO2, 19:1). The cell suspension was centrifuged at 50 g for 5 min and the supernatant was discarded. Cells were then resuspended in Krebs bicarbonate buffer containing 2.55 mM-Ca2+ and 0.5 mg collagenase/ml in a 150 ml conical flask. The mixture was incubated at 37 °C in a shaking water bath (100

cycles/min) under continuous gassing. After 60 min, the cell suspension was washed with Krebs bicarbonate buffer containing 2.55 mM-Ca2+ and then centrifuged at 35 g for 5 min and filtered through a nylon mesh (500 µm). The washing step was repeated with a nylon mesh of 100 µm. During washings at very low speed, separation occurred between parenchymal and haematopoietic cells, the latter mostly remaining in suspension. By counting under a microscope, haematopoietic cell contamination was shown to be lower than 5%. The procedure produced approx. 1.5 x 10^7 cells/g of fetal liver, representing about a 15% recovery yield. Cell viability (Trypan blue exclusion) for fetal hepatocytes was always higher than 95%.

Cell culture

For culture of cells sterile techniques were used throughout the procedure and media was supplemented with 120 µg penicillin-G/ml, 100 ug streptomycin/ml. The isolated cells were plated in 100 mm diameter plastic dishes containing 8 ml medium 199 with Earle's salts supplemented with 10% (v/v) fetal calf serum and antibiotics as described above. Each dish was inoculated with 3-4 x10^6 cells and the primary culture was kept at 37 °C under an atmosphere of 5% CO2 in air with 80% humidity in a cell incubator for 4-5 h. Then, the attached monolayer of cells were washed twice with serum-free medium and fresh serum-free medium supplemented with the various different conditions was added and the dishes incubated for the indicated times. The use of this procedure ensures a fairly pure culture of fetal hepatocytes in which the fibroblast-like cells comprise less than 10% of the total cells (6).

Preparation of RNA

Cultured hepatocytes were separated from the plastic substrate with a rubber policeman and total RNA was prepared by homogenization of cells in guanidinium thiocyanate as originally described (7). RNA was re-precipitated for purification and its concentration determined by absorbance at 260 nm. Samples were electrophoresed through 1.1% agarose, 2.2 mol formaldehide/l gels and stained with ethidium bromide in order to render the 28S and 18S ribosomal RNA visible and thereby confirm the integrity of the RNA and normalize the quantity of RNA in the different lanes. A beta-actin probe (0.6 Kb EcoRI/HindIII fragment isolated from the VC18 vector kindly provided by Dr. P. Martín-Sanz from Inst. Bioq. CSIC, Madrid, Spain) was used in a Northern blot assay in order to validate the ethidium bromide method for loading normalization.

Riboprobes

Rat IGF-I, IGF-II and IGFBP3 cDNAs were kindly provided by Dr. E. Hernández (Inst. Bioq. CSIC, Madrid, Spain), Dr. C. T. Roberts and Dr. D. LeRoith (NIH, Bethesda, MD, USA). Rat IGF-I cDNA ligated into a pGEM-3 plasmid (Promega Biotech, Madison, WI) was linearized with HindIII and an antisense riboprobe was produced by T7 RNA polimerase generating two protected fragments of 224 bases (Ia) and 386 bases (Ib). Rat IGF-II cDNA ligated into a pGEM-3 plasmid was linearized with HindIII and incubated with T7 RNA polimerase to generate a riboprobe that recognized a protected fragment of 500 bases. Rat IGFBP3 cDNA ligated into a pGEM-4Z plasmid was linearized with Acc I, and use was made of T7 RNA polymerase to generate a 343-base-long antisense riboprobe.

Solution hybridization/RNAse protection assay

Solution hybridization/RNAse protection assays were performed as previously described (8). Briefly, 20 μg of total liver RNA were hybridized with 500,000 cpm of the 32P-labeled riboprobes described above for 18 hours at 45 °C in 75% formamide and 400 mmoles NaCl/l. After RNAse digestion with a buffer containing 40 μg RNAse A/ml and 2 μg RNAse T1/ml for 1 h at 37 °C, protected RNA-RNA hybrids were resolved on denaturing 8% polyacrylamide and 8 moles urea/l gels. Autoradiography was performed at -70 °C against an Hyperfilm MP film between intensifying screens.

RESULTS

Effect of glucose and GH on IGF-I gene expression:

No significant effect on IGF-I mRNA expression was observed after treatment of the culture with several increasing concentrations of glucose (figure 1A).

While different doses of GH (10-500 ng/ml) evoked a significant increase of IGF-I and IGFBP3 gene expression in cultures of adult hepatocytes, only a slight induction of IGF-I gene expression was observed in cultures of fetal hepatocytes treated with the same GH concentrations (figure 1B and C).

Effect of glucose, serum and GH on IGF-II gene expression:

As in the case of IGF-I, glucose (10-100 mM) addition to the medium did

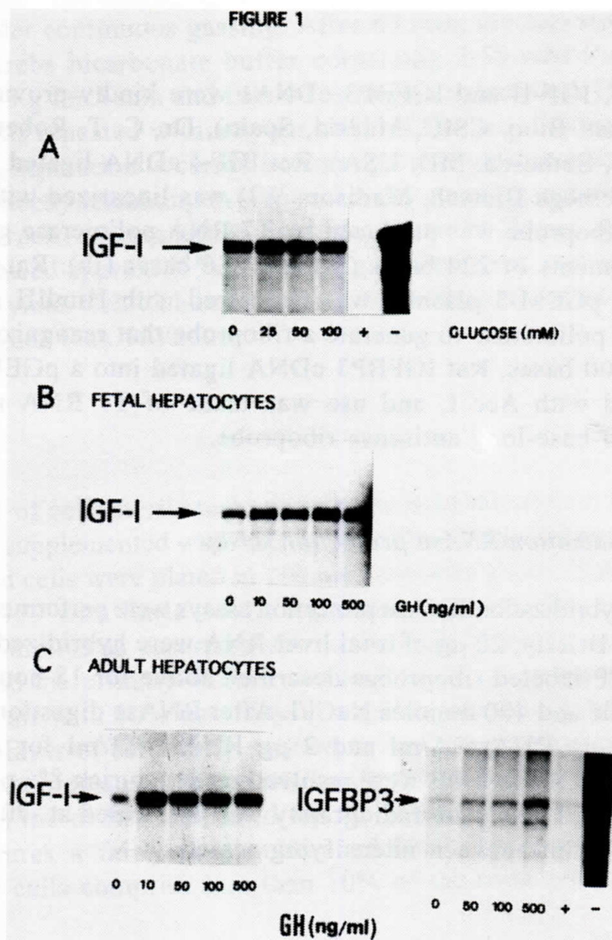

Figure 1.—mRNA expression levels of IGF-I and IGFBP3 in primary cultures of hepatocytes.

A) RNAse protection assay of IGF-I mRNA transcripts in cultures of hepatocytes from fetuses on day 21 of gestation treated with three different doses of glucose (25-100 mM). + and - designate riboprobe lanes treated with or without RNases, respectively.

B) RNAse protection assay of IGF-I mRNA transcripts in cultures of fetal hepatocytes treated with four different doses (10-500 ng/ml) of growth hormone (GH).

C) RNAse protection assay of IGF-I (left pannel) and IGFBP3 (right pannel) mRNA transcripts in cultures of adult hepatocytes treated with different doses of GH (10-500 ng/ml). + and - designate riboprobe lanes treated with or without RNases, respectively.

not evoke significant changes in the abundance of IGF-II mRNA expression (figure 2A). On the other hand, the presence of 10% fetal calf serum in the medium induced a rapid significant increase in IGF-II gene expression 1 h after the onset of culture and maintained elevated IGF-II gene expression

levels at 3 h of culture (figure 2B, left pannel), an effect directly dependent on serum factors and not on the amount of protein present in the medium as demonstrated by the lack of effect of addition of an equivalent amount of bovine serum albumin (figure 2B, right pannel).

Although treatment of the cultures with several doses of glucose revealed no significant effect on IGF-I or -II gene expression (figures 1A and 2A), a permissive role of insulin and other serum factors mediating glucose metabolism in the cultured fetal hepatocyte needed to be discarded by using a glucose-free medium.

As in the presence of glucose, 10% serum maintained higher IGF-II gene expression levels throughout time in cultures of hepatocytes in a glucose-free medium (figure 2C, left pannel), while no significant induction was observed after addition to the medium of an equivalent amount of bovine serum albumin (data not shown). As expected, no significant effect after treatment with GH for 20 h was observed in cultures of fetal hepatocytes both in the absence (figure 2C, right pannel) and presence (data not shown) of glucose.

DISCUSSION

Previous reports from our laboratory have unequivocally shown that IGFs regulation during fetal life and early stages of neonatal development is GH-independent (2,3). Moreover, nutritional status and serum insulin concentration are the main factors involved in the regulation of IGFs synthesis and secretion during the perinatal period of the rat (2,3). These *in vivo* experimental models have been widely used for the study of IGF regulation by the nutritional status and insulin concentration and have shown the relative contribution of factors, other than GH, involved in liver IGF synthesis and secretion. However, the study of the molecular pathways and mechanism of action of the different factors on the IGF regulation may benefit from the establishment of experimental models *in vitro* where selective conditions may unravel the specific or non-specific effect of such factors. The study of the effect and mechanism of action of regulatory factors on IGF synthesis *in vitro* has been carried out mainly in cultures of adult hepatocytes, which have been shown to produce IGF-I (9,10,11) as well as IGFBPs (12,13). Different nutrients (including amino acid availability), hormones and metabolites have been demonstrated as putative regulatory factors on this system (1,11,14,15).

The factors involved in the IGF regulation seem to be the same during the lifetime although the relative contribution is different depending on the stage of development (16). The study of the IGF regulation is particularly interesting in stages of immaturity when such regulation is GH-independent and other

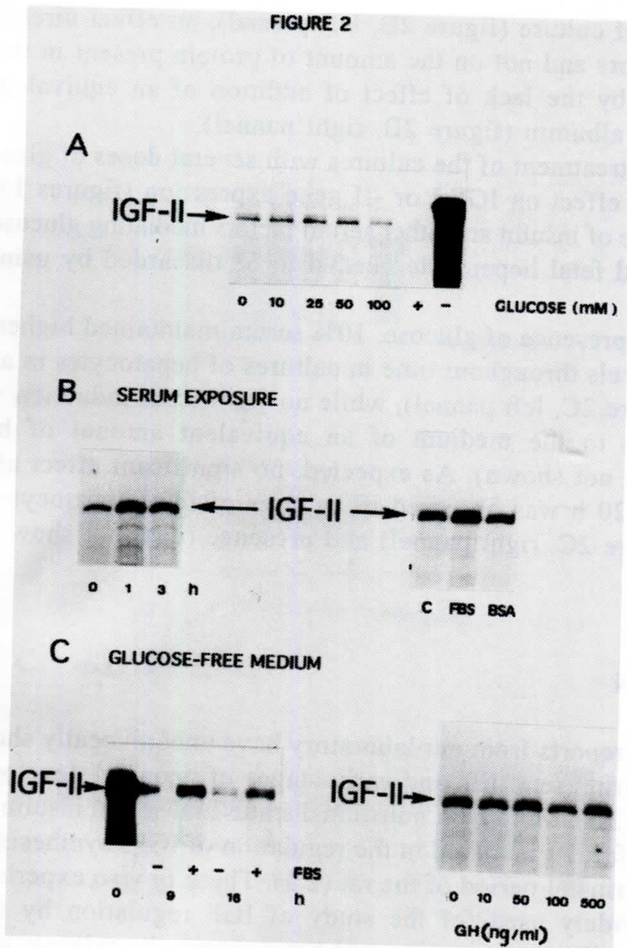

Figure 2.—Liver mRNA expression levels of IGF-II in cultures of fetal hepatocytes.

A) RNAse protection assay of IGF-II mRNA transcripts in cultures of hepatocytes from fetuses on day 21 of gestation treated with four different doses of glucose (10-100 mM). + and - designate riboprobe lanes treated with or without RNases, respectively.

B) RNAse protection assays of IGF-II mRNA transcripts in cultures of fetal hepatocytes treated with 10% fetal bovine serum. Left pannel: time-course effect of serum exposure; right pannel: effect of 20 h treament with 10% fetal bovine serum (FBS) and 100mg/ml bovine serum albumin (BSA).

C) RNAse protection assays of IGF-II mRNA transcripts in glucose-free cultures of fetal hepatocytes.
Left pannel: time-course IGF-II mRNA expression in the absence (-S) and presence (+S) of 10% fetal bovine serum. Right pannel: mRNA transcripts of IGF-II in cultures of fetal hepatocytes treated with different doses of GH (10-500 ng/ml).

factors may have a decisive role. However, research on IGFs and IGFBPs synthesis and secretion by fetal hepatocyte cultures is scant mainly due to changes in differentiating pattern and a very low synthesis of these peptides by fetal hepatocytes in plastic substratum. To overcome these difficulties we have established short term cultures of 24 h preventing loss of IGF synthesis and we have made use of the highly sensitive RNase protection assay to determine mRNA transcript expression of IGF-I and -II. The primary culture of late fetal hepatocytes described in this article is an attempt to deliniate direct fròm indirect unspecific effects of factors such as glucose, GH and serum factors on the regulation of liver mRNA synthesis of IGFs during stages of development. The results obtained in these conditions show that fetal hepatocytes cultured for short periods in plastic substratum produce IGF-I and -II and that their gene expression is regulated by the presence of serum in the culture medium.

Serum contains a number of hormones and growth factors that might stimulate IGF-II gene expression. Although no serum-response element has been reported in the IGF-I and -II promoters at present, our results show that the presence of 10% fetal calf serum rapidly induces and maintains IGF-II transcript expression levels; thus, in order to investigate the specific effect of glucose and GH, the fetal hepatocytes were cultured in serum-free medium supplemented with the conditions to study. Treatment of the cultures with several doses of glucose (10-100 mM) showed no significant effect on IGF-I or -II gene expression, but the presence of glucose in the culture medium could suggest a possible indirect effect of serum factors, including insulin, mediating glucose metabolism in the cultured fetal hepatocyte (17). This permisive role was discarded since serum stimulated IGF-II gene expression in a glucose-free medium. By using the same serum-free conditions GH did not significantly affect IGF-I nor -II gene expression in fetal hepatocyte cultures, while GH induced a consistent increase in IGF-I transcripts in cultures of adult hepatocytes, supporting the GH-independent regulation of IGFs at stages of development already demonstrated *in vivo* by us (2,3) and other authors (18).

In summary, the results obtained in a primary culture of fetal hepatocytes strongly suggest the regulatory role of serum factors on IGF-I and -II gene expression during stages of perinatal development. The results also demonstrate the successful isolation of fetal rat hepatocytes that retain some of the characteristics of the rat fetal liver while maintained in short term culture. Finally, these results have raised two immediate goals already in progress in our laboratory: (a) study of the effect of insulin and glucocorticoids on IGF-I and -II synthesis and (b) study of IGFBPs regulation on fetal hepatocyte cultures.

ACKNOWLEDGMENTS

This work was supported by DGICYT (Dirección General de Investigación, Ciencia y Tecnología), Ministerio de Educación y Ciencia, España, ref. PM 92-0249.

REFERENCES

(1) THISSEN, J. P., KETELSLEGERS, J. M. and UNDERWOOD, L. E.: "Nutritional regulation of the insulin-like growth factors". *Endocrine Rev* (1994), **15**: 81-101.

(2) RIVERO, F., GOYA, L., ALÁEZ, C. and PASCUAL-LEONE, A. M.: "Effects of undernutrition and diabetes on serum and liver mRNA expression of IGFs and their binding proteins during rat development". *J Endocrinol* (1995), **145**: 427-440.

(3) GOYA, L., RIVERO, F., MARTÍN, M. A., ARAHUETES, R., HERNÁNDEZ, E. R. and PASCUAL-LEONE, A. M.: "Effects of refeeding of undernourished and insulin treatment of diabetic neonatal rats on serum content and liver mRNA expression of IGFs and IGFBPs". *Am J Physiol* (1996), **271**: E223-E231.

(4) GOLDSTEIN, S., HARP, J. B. and PHILLIPS, L. S.: "Nutrition and somatomedin. XXII. Molecular regulation of insulin-like growth factor-I during fasting and refeeding in rats". *J Mol Endocrinol* (1991), **6**: 33-43.

(5) LORENZO, M., RONCERO, C. and BENITO, M.: "The role of prolactin and progesterone in the regulation of lipogenesis in maternal and foetal rat liver *in vivo* and in isolated hepatocytes during the last day of gestation". *Biochem J* (1986), **239**: 135-139.

(6) RONCERO, C., LORENZO, M., FABREGAT, I. and BENITO, M.: "Rates of lipogenesis in fetal hepatocytes in suspension and in primary culture: hormonal effects". *Biochim Biophys Acta* (1989), **1012**: 320-324.

(7) CHOMCZYNSKI, P. and SACCHI, N.: "Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction". *Anal Biochem* (1987), **162**: 156-159.

(8) LOWE, W. L., ROBERTS, C. T., LASKY, S. R. and LEROITH, D.: "Differential expression of alternative 5'untranslated regions in mRNAs encoding rat insulin-like growth factor I". *Proc Nat Acad Sci USA* (1987), **84**: 8946-8950.

(9) BONI-SCHNETZLER, M., SCHMID, C., MEIER, P. J. and FROESCH, E. R.: "Insulin regulates insulin-like growth factor I mRNA in rat hepatocytes". *Am J Physiology* (1991), **260**: E846-E851.

(10) PHILLIPS, L. S., GOLDSTEIN, S. and PAO C.-I.: "XXI. Molecular regulation of IGF-I by insulin in cultured rat hepatocytes". *Diabetes* (1991), **40**: 1525-1530.

(11) ARANY, E., STRAIN, A. J., HUBE, M. J., PHILLIPS, I. D. and HILL, D. J. "Interactive effects of nutrients and hormones on the expression of insulin-like growth factor binding protein-1 (IGFBP1) mRNA and peptide, and IGF-I release from isolated adult rat hepatocytes". *J Cell Physiology* (1993), **155**: 426-35.

(12) VILLAFUERTE, B. C., GOLDSTEIN, S., MURPHY, L. J. and PHILLIPS, L. S.: "XXV. Regulation of insulin-like growth factor binding protein 1 in primary cultures of normal rat hepatocytes". *Diabetes* (1991), **40**: 837-841.

(13) VILLAFUERTE, B. C., KOOP, B. L., PAO, C.-I., GU, L., BIRDSONG, G. G. and PHILLIPS, L. S.: "Coculture of primary rat hepatocytes and nonparenchymal cells permits expression of insulin-like growth factor binding protein-3 *in vitro*". *Endocrinol* (1994), **134**: 2044-2050.