# ONTOGENY AND EFFECT OF WEANING ON mRNA LEVELS OF IGFs, EGF AND bFGF IN VARIOUS TISSUES OF THE PIG

By

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*To my motherland P.R. China*

## RÉSUMÉ

Ce travail porte sur l'étude de trois facteurs de croissance qui peuvent être importants dans le développement d'organes particuliers du porc soit le pancréas, le foie, les reins et le muscle strié. On croit que ces facteurs de croissance seraient directement impliqués dans la croissance de ces tissus. Ces trois facteurs sont le facteur de croissance insulinique -I et -II (IGFs), le facteur de croissance épidermique (EGF) et le facteur de croissance fibroblastique basique (bFGF). Nous avons donc mesuré les niveaux d'ARN messager (ARNm) de chacun des facteurs et de leurs récepteurs (R) ainsi que les protéines liantes de IGF (IGFBPs) à partir du stade foetal jusqu'à l'âge de 180 jours. Dans le cas des IGF et de ses protéines liantes, les concentrations sériques et dans chacun des tissus ont aussi été mesurées.

Les concentrations sériques d'IGFs et d'IGFBPs croissent avec l'âge, l'IGFBP-3 étant la forme dominante lors de la période postnatale. Dans le tissu pancréatique, on observe des niveaux d'ARNm de l'IGF-I qui sont très élevés dans le pancréas foetal et à la naissance. Au début du sevrage (21$^{ième}$ jour), la concentration d'un des deux types d'IGF, soit l'IGF-I, augmente de façon importante, y atteignant un maximum. Cette augmentation est concomitante avec un niveau élevé de son ARNm et des zymogènes pancréatiques. Quant aux niveaux pancréatiques des ARNm de l'IGF-II et du récepteur de l'IGF-I (IGF-IR), ils suivent un patron similaire à la concentration d'IGF-II, laquelle est élevée chez le foetus de 90 jours, période qui correspond à une croissance rapide du pancréas. Les niveaux d'ARNm

de l'EGF, de son récepteur (EGFR) et du bFGF sont plus élevés durant la vie foetale qu'après la naissance. Ces observations suggèrent que les quatre facteurs de croissance étudiés sont impliqués dans le développement pancréatique foetal. À l'âge de 27 et 30 jours, on a constaté que les niveaux d'ARNm pancréatique de l'EGF sont plus élevés chez les porcelets sevrés que chez ceux qui sont encore allaités, ce qui suggère un rôle de l'EGF dans le développement pancréatique lors du sevrage.

Dans le foie, les niveaux d'ARNm de l'IGF-I sont parallèles à sa concentration dans le tissu. Les concentrations élevées de l'IGF-I et -II dans le tissu correspondent à des périodes de croissance accélérée de l'organe, soit entre le 1$^{er}$ et le 21$^{ième}$ jour. Par comparaison avec les autres tissus étudiés, c'est dans le foie que l'ARNm de l'IGFBP-I est le plus élevé. Bien que le niveau d'ARNm du récepteur de l'EGF soit élevé, l'ARNm de l'EGF lui-même n'a pu y être détecté. Quant au bFGF hépatique, ses niveaux d'ARNm sont élevés durant toute la période de développement.

Dans le rein, les niveaux d'ARNm des IGFs étaient élevés dans le foetus tandis que les concentrations des IGFs atteignaient un maximum chez le nouveau-né, lesquelles étaient associées à une période de croissance rapide de cet organe. Comme dans le foie, les ARNm du bFGF sont abondants mais stables contrairement au niveau d'ARNm de l'EGF qui augmente durant cette période de développement.

Dans le muscle strié, les ARNm des deux espèces d'IGF (IGF-I et IGF-II), de son récepteur, de ses protéines liantes ainsi que le bFGF sont abondants durant toute la période foetale et les premiers jours de vie. Quant à l'EGF, son niveau d'ARNm y augmente tout

au cours du développement. Ces observations suggèrent donc que les IGF, l'EGF et les bFGF sont des régulateurs physiologiques de la croissance et de la maintenance du tissus musculaire strié.

On peut donc conclure que les niveaux d'ARNm et les concentrations dans les tissus des différents facteurs de croissance étudiés ici et ceux de leur protéines accessoires varient considérablement selon l'organe et l'âge de l'animal. Nous avons mis en évidence que les ARNm des IGF, de leurs récepteurs et de leurs protéines liantes sont parfois contrôlés de façon coordonnée dans les tissus comme le pancréas et le foie. Nous avons aussi mis en évidence que les périodes de croissance rapide du pancréas, du foie et du rein sont accompagnées par des niveaux élevés d'ARNm des IGF, de l'EGF et du bFGF, suggérant donc que ces facteurs de croissance sont d'importants régulateurs de la croissance et du développement de ces organes.

## SUMMARY

In the present study, we have examined three growth factors which can be important for organ development of pancreas, liver, kidney and muscle. We believe that these growth factors are involved in the growth of these tissues. These growth factors are the insulin like growth factor-I and -II (IGFs), the epidermal growth factor (EGF) and the basic fibroblast growth factor (bFGF). We have measured the messenger RNA (mRNA) levels of these growth factors and those of their receptors (R) as well as IGF binding proteins (IGFBPs) from fetal life to 180 days of age. In the case of IGFs and IGFBPs, concentrations in the serum and in each tissue were also measured.

The IGFs and IGFBPs serum concentrations increased with advancing age, the IGFBP-3 being the major IGF binding protein in serum during postnatal life. In the pancreas, IGF-I mRNA levels were high during the fetal and early postnatal life. Around the weaning period (21 days of age), tissue concentration of IGF-I reached a maximal level which was concomitant with a high IGF-I mRNA level and an increased activity of pancreatic enzymes. Pancreatic IGF-II and IGFI-R mRNA levels as well as IGF-II tissue concentrations were high in fetuses and were accompanied by a rapid growth period of pancreas in fetuses of 90 days of age. Levels of EGF, EGFR and bFGF mRNAs were higher during the fetal life than after birth. These results imply that multiple growth factors are involved in fetal pancreatic development. At 27 and 30 days of age, EGF mRNA levels were higher in weaned than in suckling piglets, suggesting a possible involvement of EGF in pancreatic development during

the weaning period.

In the liver, IGF-I mRNA level paralleled its tissue concentration. High tissue concentration of both IGF-I and -II at 1 and 21 days of age corresponded to an accelerated growth period of the liver. The IGFBP-1 mRNA level was the most abundant in the liver when compared to that of other tissues. In the liver, abundant EGFR mRNA level was observed whereas EGF mRNA was undetected. The bFGF mRNA levels were high over the whole developmental period.

In the kidney, IGFs mRNA levels were high in fetuses while kidney IGFs concentrations peaked in newborns, which were associated with a fast growth period of this organ. As observed in the liver, the bFGF mRNA was abundant in the kidney during the whole developmental period, while EGF mRNA level increased with development.

In the skeletal muscle, IGF-I, IGF-II, IGF-IR, IGFBP-3 and bFGF mRNAs were all abundant during fetal and neonatal life. The EGF mRNA level increased during development. These data suggest that IGFs, EGF and bFGF are likely physiological regulators of growth and maintenance of the skeletal muscle.

In conclusion, mRNA levels and concentrations of growth factors and their binding proteins are tissue and developmentally regulated. Concentrations and mRNA levels of IGFs, IGFRs and IGFBPs are coordinately regulated in the pancreas and liver. Fast growth periods of pancreas, liver and kidney are accompanied by high concentrations of IGFs and abundant mRNA levels of IGFs, EGF and bFGF, suggesting that these growth factors are important regulators for growth and development of these organs.

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## CONTENTS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AC: | adenylate cyclase |
| ALS: | acid-labile |
| bFGF: | basic fibroblast growth factor |
| BSA: | bovine serum albumin |
| cAMP: | cyclic adenosine monophosphate |
| CBP: | CREB-binding protein |
| CCK: | cholecystokinin |
| cDNA: | complementary DNA |
| CKII: | casein kinase II |
| CRE: | cAMP-response element |
| CREB: | cAMP-response element-binding protein |
| CRID: | coding region instability determinant |
| CTD: | C-terminal domain |
| DAG: | diacylglycerol |
| DNA: | deoxyribonucleic acid |
| EDTA: | disodium ethenediamine tetraacetic acid |
| EGF: | epidermal growth factor |
| EGFR: | EGF receptor |
| FRK: | Fos-regulating kinase |

| | |
|---|---|
| GH: | growth hormone |
| GHR: | GH receptor |
| GAP: | GTPase-activating protein |
| GDP: | guanosine diphosphate |
| GTP: | guanosine triphosphate |
| HGF: | hepatocyte growth factor |
| HLH: | helix-loop-helix |
| IGF: | insulin-like growth factor |
| IGFBP: | IGF binding protein |
| IGFR: | IGF receptor |
| IkB: | inhibitor protein |
| IP3: | inositol 1,4,5-trisphosphate |
| IRS: | insulin-receptor substrate |
| JAK: | Janus kinase |
| JNK: | c-Jun $NH_2$-terminal kinase |
| MAPK: | mitogen activated protein kinase |
| MAPKK: | MAPK kinase |
| mRNA: | messenger RNA |
| NC: | negative cofactor |
| NF-I: | neurofibromatosis type I protein |
| ODC: | ornithine decarboxylase |

| | |
|---|---|
| PABP: | poly (A)-binding protein |
| PH: | pleckstrin homology |
| PI3-K: | phosphoinositide 3-kinase |
| PIP2: | phosphatidylinositol 4,5-bisphosphate |
| PKC: | protein kinase C |
| PL: | phospholipase |
| PLD: | phospholipase D |
| PLC: | phospholipase C |
| PLC-$\gamma$: | phospholipase C-$\gamma$ |
| PLC-$\beta$: | phospholipase C-$\beta$ |
| Pol II: | polymerase II |
| RIA: | radioimmunoassay |
| RNA: | ribonucleic acid |
| RTK: | receptor tyrosine kinase |
| RT-PCR: | reverse transcription polymerase chain reaction |
| SDS: | sodium dodecyl sulfate |
| SH2: | src homology 2 domains |
| SP1: | stimulated protein 1 |
| SRE: | serum response element |
| SRF: | serum response factor |
| STAT: | signal transducer and activator of transcription |

TBP: TATA box binding protein

TBS: tris buffer saline

TCF: ternary complex factor

TPA: 12-0-tetradecanoyl-phorbol-13-acetate

TRE: TPA response element

TF: transcription factor

TTR: transthyretin

USF: upstream stimulatory factor

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# CHAPTER I INTRODUCTION

## PART A: BASIC KNOWLEDGE

### 1.1 EMBRYONIC DEVELOPMENT OF PANCREAS, LIVER, KIDNEY AND SKELETAL MUSCLE IN THE HUMAN

The first phase of human embryonic development lasts 8 weeks. From week 9 of gestation starts the fetal period which can be subdivided into the previable fetal period (9-18 weeks of development) and the viable fetal period (19-38 weeks of development). Development during the whole fetal period primarily concerns rapid body growth and further differentiation of organs and tissues (Kalousek et al., 1990).

*Pancreas*

The pancreatic exocrine and endocrine tissues arise from the dorsal and ventral pancreatic buds that originate from the endodermal lining of the foregut (Gray et al., 1987). The pancreas is first detectable during the fifth week of gestation. By the 17th week, as a consequence of rotation and fixation of the gut, the two buds fuse to form definitive ducts (Fig.1.1.1). The dorsal bud contributes to the tail and body of the mature gland, while the ventral bud contributes to the head. The main pancreatic duct is formed by the fusion of the distal part of the dorsal pancreatic duct and the entire ventral pancreatic duct. The accessory pancreatic duct is derived from the proximal dorsal pancreatic duct (Fig.1.1.1). By 12 to 14

weeks, tubules become a network of interconnected ductulus and separation of endocrine from exocrine tissue has occurred. Discrete lobules and acini can be identified by 14-16 weeks. By 20 weeks, the acinar cells are well polarized with many mature zymogen granules crowded in the apical cytoplasm (Table 1.1.1 and 1.1.2).

*Liver*

The liver bud is formed from outgrowth of the endodermal epithelial lining of the foregut early in the fourth week (Kalousek et al., 1990). The caudate lobe and the quadrate lobes develop as a subdivision of the right lobe. The hepatic volume consistently increases during embryonic life. At about 8 to 9 weeks, the liver represents 10% of the body weight due to rapid growth and erythroblast accumulation. By term, the weight of the liver is only 5% of the total body weight. During the early fetal stage, the basic glandular unit of the liver, seen through childhood and adulthood, is established. This unit, called the hepatic triad, consists of the bile ducts, hepatic artery, and portal vein. Differentiating biliary ducts can be seen in the connective tissue at 9 to 12 weeks. Bile formation by hepatic cells begins during the 12th week.

*Kidney*

The kidney begins to develop early in the 5th week and start to function about six weeks later (Kalousek et al., 1990). Kidney develops from the nephrogenic blastema and ureteric bud. The nephrogenic blastema gives rise to the nephrons each consisting of a glomerulus and a proximal convoluted tubule, a loop of Henle, and a distal convoluted tubule. The ureteric bud differentiates into collecting tubules, renal pelvis, calyces, and

ureter. Glomeruli are first formed in the region of the metanephric tissue that, in the definitive kidney, constitutes the juxtamedullary region. It has been estimated that approximately 20% of nephrons are morphologically formed and mature at 9 to 11 weeks of development, and another 10% is added at 14-18 weeks. By 12 weeks, the kidney is differentiated into a cortex and a medulla. By 16 weeks, development of the pyramids is advanced. Glomeruli continue to form from the metanephric blastemal cap until about 36 weeks of gestation.

*Muscle*

The muscle system develops from the mesoderm (Hauschka, 1994). Muscle tissue develops from primitive cells called myoblasts which are derived from mesenchyme. The first indication of muscle development is the elongation of the nuclei and cell bodies of the mesenchymal cells to form myoblasts. Soon, these cells begin to fuse with one another to form elongated, multinucleated, cylindrical structures called myotubes. Growth occurs by continued fusion of myoblasts and myotubes. The specialized myofilaments develop in the cytoplasm of the myotubes during or after fusion of the myoblasts. Then, the myofibrils and other organelle characteristics of striated muscle fibres (cells) develop. Most striated skeletal muscle fibres develop before birth, and almost all the remaining ones are formed by the end of the first year. After birth, muscle increases in length and width in order to grow with the skeleton.

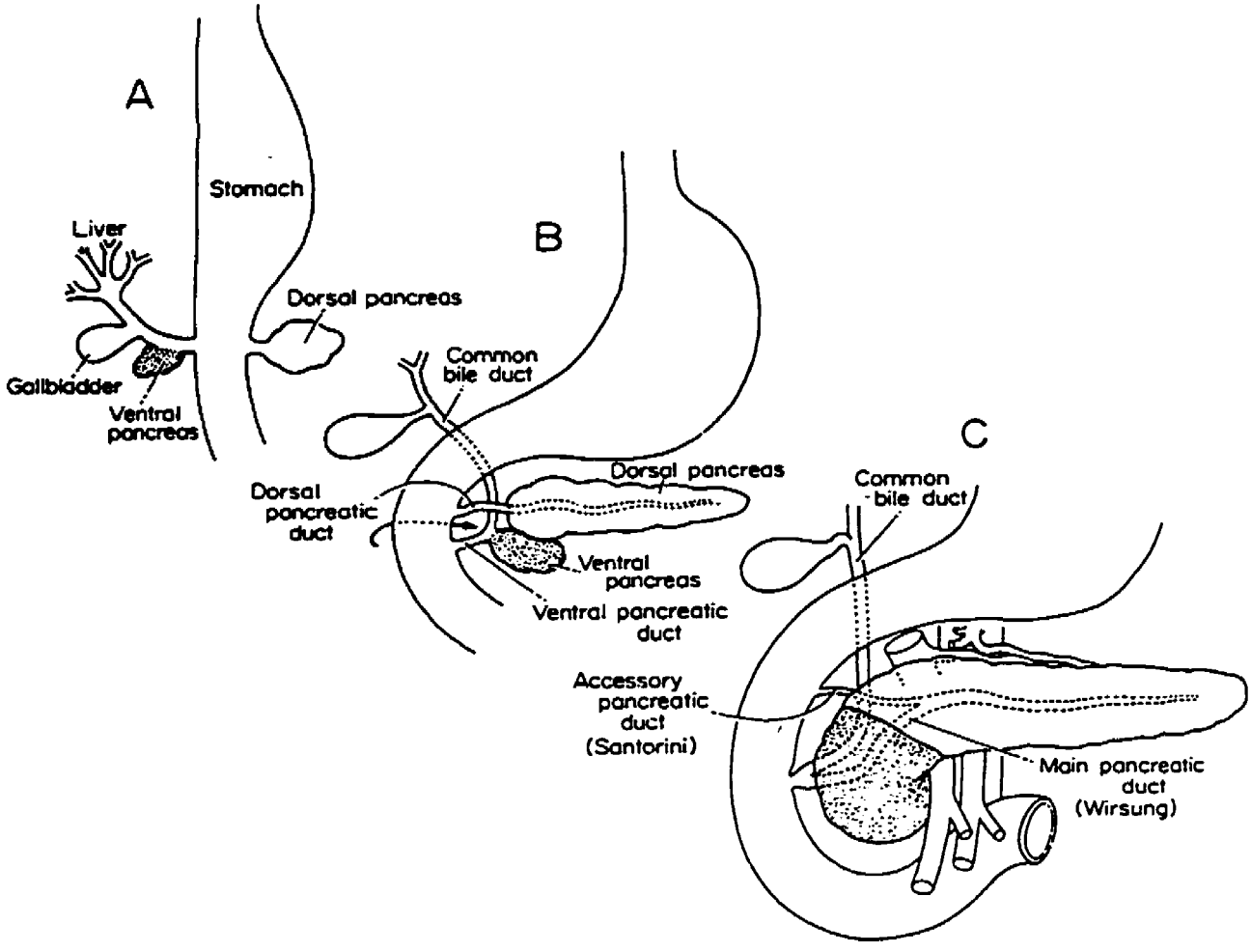


Fig. 1.1.1 Development of pancreas in human embryo during the fourth to sixth week of gestation. A. Formation of the dorsal and ventral pancreatic primordia. B. Rotation of the ventral primordium. C. Fusion of the primordia and formation of the definitive ducts. The contribution of the ventral primordium is shaded. (From Gray, S.W., Skandalakis, J.E. and Skandalakis, L.J. In: Surgical diseases of the pancreas, pp. 37-45, 1987).

Table 1.1.1 Morphological development of human exocrine pancreas

| Event | Developmental age (weeks) |
|---|---|
| Dorsal and ventral anlagen detectable | 5 |
| Fusion of anlagen | 7 |
| Differentiation of endocrine and exocrine tissue | 12-14 |
| Lobules and acini formed | 14-16 |
| Polarity of acinar cells established | 20 |
| Zymogen granules present | 20-22 |
| Adult appearance of gland (histological) | 40 |

Table 1.1.2 Functional development of human exocrine pancreas

| Event | developmental age |
|---|---|
| Zymogens detectable in pancreatic homogenates | 20 weeks |
| Lipase detectable in pancreatic homogenates | 24 weeks |
| Enterokinase detectable in intestinal content | 26 weeks |
| Response to secretagogues | 1 month (postnatal) |
| Amylase detectable in pancreatic homogenates | 2 months (postnatal) |

## 1.2 SIGNAL TRANSDUCTION PATHWAYS AND CELL PROLIFERATION

Cell proliferation is controlled by a large variety of stimulative or inhibitory signals mediated through specific receptors on the cell surface. One class of receptors consists of transmembrane surface receptors with intrinsic tyrosine kinase activity, some of which bind polypeptide growth factors such as IGF-I, EGF and FGF (Geer and Hunter, 1994). The second class of receptors consists of G protein-coupled receptors interacting with different intracellular signalling systems (e.g. phospholipid breakdown, adenylyl cyclase, ion channels) and bind different hormones, such as thrombin, serotonin and bombesin (Strader et al., 1994). These two classes of receptors are tightly interconnected.

*Signal transduction by receptors with ligand-dependent tyrosine kinase activity*

Polypeptide growth factors represent a group of extracellular signals that are critically important for influencing a diverse array of cellular responses including proliferation, differentiation and cell survival. The effects of many growth factors are mediated by high-affinity tyrosine kinase receptors (Geer and Hunter, 1994). Ligand binding to receptor tyrosine kinase results in dimerization of adjacent receptor monomers and activation of tyrosine kinase phosphorylation, a process termed autophosphorylation. Receptor autophosphorylation acts as a switch to induce the binding of cytoplasmic signal proteins to the receptor. These signalling molecules mediate the cellular responses to growth factors. Over the recent years, several tyrosine kinase substrates and cell mediators have been

identified, including phospholipase C-$\gamma$ (PLC-$\gamma$), phosphoinositide-3-kinase (PI-3K), insulin-receptor substrate (IRS-1) and GTPase-activating protein (GAP) modulating c-ras proto-oncogene function (Pawson, 1992). All these tyrosine kinase substrates contain specific protein sequences Src homology 2 domains or Src homology 3-like region (SH2 or SH3 domains) which allow them to associate with specific tyrosine phosphorylated sites on receptor proteins.

PLC activation is a common feature in the mechanism of action of a wide variety of growth factors and mitogens. Several PLC isozymes can mediate this step. G-protein coupled receptors activate PLC-$\beta$, while tyrosine kinase receptors other than insulin and IGF-I receptors utilize PLC-$\gamma$ as a substrate. Hydrolysis of the PLC substrate phosphatidylinositol 4,5-bisphosphate (PIP2) produces two second messengers, diacylglycerol (DAG) which activates protein kinase C (PKC), and inositol 1,4,5-trisphosphate (IP3) which mobilizes intracellular stored calcium.

PI-3-kinase is another tyrosine kinase receptor substrate involved in phosphoinositide metabolism. PI-3-kinase phosphorylates phosphatidylinositol at position 3 of the inositol ring to produce PI-3-P (which is not a substrate for PLC). PI-3-P is an important mediator of signal transduction, linked to several biological actions during growth factor stimulation.

IRS-1 is the principal substrate of insulin and IGF-I receptor tyrosine kinases (Myers et al., 1994). The activated receptor physphorylates IRS-1 and SH2 containing proteins bind to IRS-1. IRS-I contains a pleckstrin homology (PH) domain which mediates the interaction of IRS-1 with a common motif in the receptors for insulin and IGF-I. Removal of the PH

domain from IRS-1 impairs insulin signalling in a background of low insulin receptor expression. IRS-1 mediates cellular mitogenesis during stimulation with insulin and IGF-I. Overexpression of IRS-1 in Chinese hamster ovary cells, which contain only a few endogenous insulin receptors, doubles the maximal response of thymidine incorporation during insulin stimulation (Sun et al., 1992).

Many studies have suggested that ras plays a key role in signal transduction and control of cellular proliferation (Marshall, 1995). Ras is active in the GTP-bound form and inactive in the GDP-bound form. The GTPase activity of ras is enhanced by GTPase-activating protein (GAP) and neurofibromatosis type I protein (NF-I). Various growth factors stimulate the formation of GTP-ras, thus coupling the signals from the cell surface to the downstream kinase cascade. There are several ras target proteins such as Raf, MEKK1 and PI3-K. Active Raf phosphorylates the MEK protein kinase on serine, which results in its activation. MEK (also known as MAP kinase kinase MAPKK) is a dual specificity threonine/tyrosine kinase that phosphorylates and activates mitogen activated protein kinase (ERK/MAPK). Although the end results of ERK substrate phosphorylations are diverse, the most significant effects on cellular proliferation are probably caused by the increased transcriptional activity of the AP-1 complex, resulting from increased expression of fos (Marais et al., 1993). ERK/MAPKs activation through Raf is the predominant ras effector pathway. MAPK functions as a key molecule in signalling processes. Active MAPK may be responsible for triggering S6 kinase and casein kinase II and may also participate in the growth factor-mediated stimulation of ion transporters such as the Na/K/Cl cotransporter or

the Na/H antiporter.

Other cytokines and growth factors function by interacting with receptors that are members of the cytokine receptor superfamily (Schindler, 1995). These receptors share the same extracellular motifs and have limited similarity in their cytoplasmic domains. Although lacking catalytic domains, in contrast to tyrosine-kinase receptors, this family of receptors couples ligand binding with the induction of tyrosine phosphorylation. Recent studies have shown that this is mediated by members of the Janus kinase (JAK) family of cytoplasmic protein tyrosine kinases (Inglese et al., 1995). Binding of a cytokine induces receptor oligomerization and activation of JAKs. The activated JAK subsequently phosphorylates both receptor and cellular substrates, which include members of the signal transducers and activators of transcription (STATs) family of transcription factors. Following phosphorylation, the STATs form dimers through SH2 domain-dependent intermolecular association with carboxyl sites of tyrosine phosphorylation. Dimerization triggers dissociation from the receptor complex and translocation to the nucleus. In the nucleus, the STAT dimers bind response elements and are generally associated with the activation of gene expression.

*Signal transduction through G protein-coupled receptors*

Many hormones, neurotransmitters and vasoactive agents transmit signals by interacting with specific G protein-coupled receptors (Strader et al., 1994). A ligand-occupied receptor induces the exchange of GDP for GTP in specific G proteins. An active G protein is a heterotrimer containing $\alpha$,$\beta$ and $\gamma$ chains. Upon activation by ligand binding,

this complex dissociates into a separate $\alpha$ subunit carrying the GTP and a $\beta\gamma$ complex. The $\alpha$ subunit will interact with effector enzymes, stimulating or inhibiting them. Several effector enzymes controlled by G proteins have been described, including adenylate cyclase (AC) and phospholipases C-$\beta$, D and $A_2$ (PLC-$\beta$, PLD and $PLA_2$). AC is the enzyme that synthesizes cAMP from ATP and is linked to G protein receptors. cAMP as a second messenger exerts its effect by activating cAMP-dependent protein kinase-A (PKA). Another effector enzyme, PLC-$\beta$, hydrolyses PIP2 into two second messengers IP3 and DAG, leading to transient rise in cytoplasmic $Ca^{++}$ levels and protein kinase C (PKC). In addition, receptor-mediated activation of PLD and PLA2 leads to hydrolysis of phosphatidylcholine into DAG and free fatty acids. A growing body of evidence now supports the idea that the $\beta\gamma$ subunit can itself interact functionally with effector proteins. It has been reported that the $\beta\gamma$ subunit can stimulate PLC$\beta$, phospholipase $A_2$ ($PLA_2$) and PI3-kinase activities and participate in the inhibition of AC. Recent studies have shown that the $\beta\gamma$ subunit may even provide a way to link G proteins to receptor tyrosine kinase (RTK) initiated cascades (Inglese et al., 1995). This connection may be mediated by $G_{\beta\gamma}$ alone or by a PH domain-directed binding, leading to ras activation. RTK activation (for example, by epidermal growth factor, EGF) also activates ras through the recruitment (directed by the SH2 and SH3 domains) of the cytoplasmic proteins Grb2 and Sos1. The convergence of these two different surface-receptor signalling pathways on ras activation, in turn, leads to the sequential activation of the raf-1-MAPK cascade.

*Signalling pathways from cell surface receptors to nuclei*

Signalling pathways from cell surface receptors to nuclei is one of the central subject of modern biology. Fig. 1.2.1 outlines some of the early events commonly activated in all cell types by different promoting agents (Hill and Treisman, 1995; Pawson, 1993). Signals through tyrosine kinase receptors and G protein-coupled receptors could converge and /or synergise at the level of switch kinases such as serine/threonine MAP kinase and Raf kinase. These kinases receive the signal from various external messengers and pass on the signals by activating other specific protein kinases (e.g. S6 kinase) to increase protein synthesis, by activating antiporter Na/H or symporter Na/K/Cl to induce ionic change, or directly by initiating early gene transcription. This notion of switch kinases although still very hypothetical, could account for the integrated and synergistic aspects of various responses.

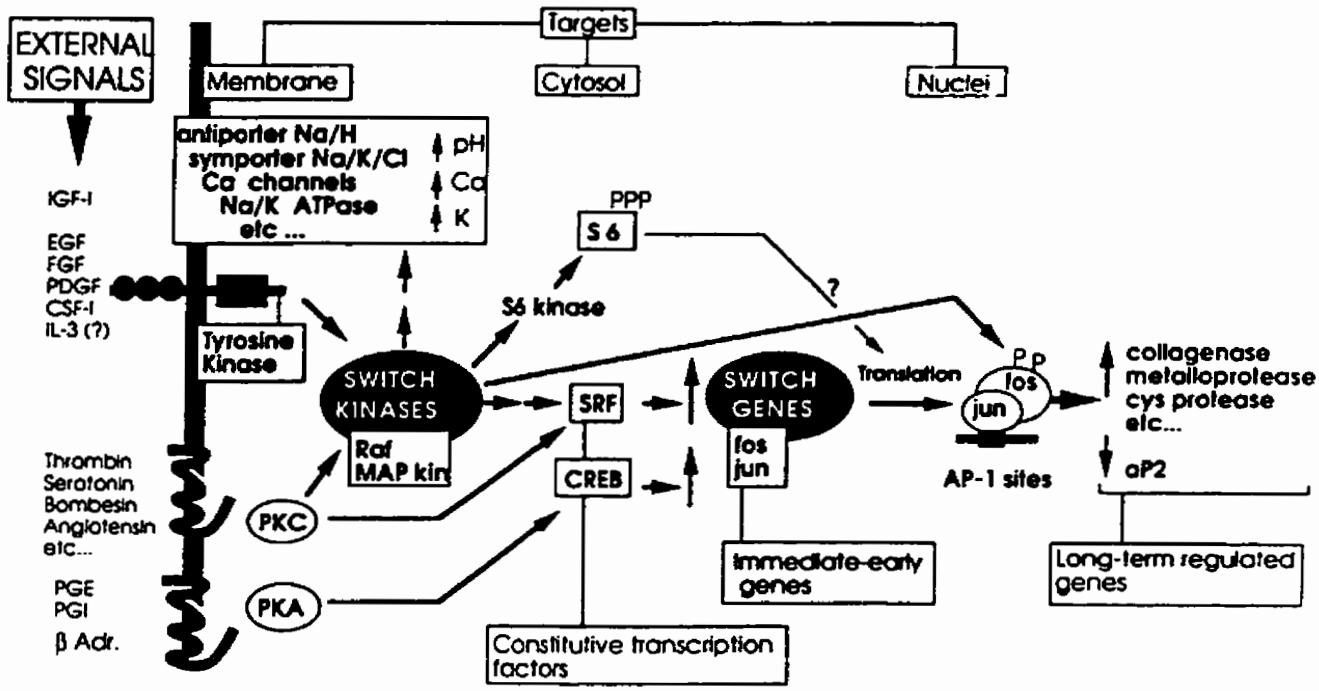


Fig. 1.2.1 Signalling pathways from the cell surface receptors to the nuclei (From Pouysségur, J., Kahan, C., Magnaldo, I. and Seuwen, K. In: Transmembrane signalling intracellular messengers and implications for drug development, pp 119-132, 1992).

## 1.3 REGULATION OF TRANSCRIPTION FACTOR EXPRESSION BY SIGNAL TRANSDUCTION PATHWAYS

Signal transduction pathways regulate nuclear transcription factors expression by modulating their activity. The most studied members are the AP-1 (Jun/Fos) and cAMP-response element-binding (CREB) protein. Molecular cloning revealed that AP-1 consists of a variety of different transcription factors which belong to the Fos and Jun families (Sassone-Corsi, 1994). Serum response element (SRE) and 12-0-tetradecanoyl-phorbol-13-acetate (TPA) response element (TRE) mediate c-Fos and c-Jun expression respectively, induction by growth factors, cytokines and other stimuli that activate MAPKs. CREB mediates transcription in response to the cAMP-dependent protein kinase A (PKA) signal transduction pathway and controls gene expression by binding to the cyclic AMP (cAMP) response element (CRE) (Brindle and Montminy, 1992).

*Altered DNA-binding and activation*

Many transcription factors bind DNA as dimers either of homodimeric or heterodimeric nature (Kerr et al., 1992). There are several different types of sequence-specific DNA-binding domains, such as zinc finger, leucine zipper/basic DNA-binding domains, helix-loop-helix (HLH) and helix-turn-helix. All members of the CREB and AP-1 families are leucine zipper containing proteins. Induction of Fos transcription results in increased synthesis of Fos proteins which combine with pre-existing Jun proteins to form

AP-1 heterodimers through leucine zipper motifs, in such a way that they bind DNA more stably and thereby increase the level of AP-1 activity.

*Signalling pathways regulating transcription factors by phosphorylation*

Regulation of transcription factor activity is mostly by phosphorylation events (Hunter, 1995). Three different types of MAPKs, the extracellular signal-regulated protein kinases (ERKs), the c-Jun $NH_2$-terminal kinases (JNKs) and the Fos-regulating kinases (FRKs) contribute to induction of AP-1 activity through phosphorylation of different substrates that potentiate their transcriptional activities. JNK-mediated phosphorylation of ATF2 and c-Jun bound to the c-Jun promoter (TRE) stimulates their transcriptional activities, leading to c-Jun production. Phosphorylation by ERK of the serum response factor (SRF) and its accessory ternary complex factor (TCF) Elk-1 bound to the c-Fos promoter serum response element (SRE) stimulates its transcriptional activity, thus leading to c-Fos induction. The newly synthesized c-Fos and c-Jun proteins combine to form stable AP-1. A further increase in AP-1 activity is brought about by the JNK and FRK which phosphorylate c-Jun and c-Fos, respectively, on sites that increase their transcriptional activities. As mentioned earlier, activation of PKA depends on increases in cAMP generated at the plasma membrane as a result of activation of adenylate cyclase by G protein-coupled receptors or other signal transduction pathways. One of the major nuclear PKA substrates is the transcription factor CREB. The PKA C subunit phosphorylates a single serine, Ser-133. This phosphorylation results in increased transactivation activity, without a significant effect on CREB DNA-

binding activity. Phosphorylation of Ser-133 promotes binding of the CREB-binding protein (CBP), a large protein with an activation domain that can interact with TFIIB (Chrivia et al., 1993).

Transcription factors can be sequestered in an inactive form in the cytoplasm. Phosphorylation of the factor itself or a cytoplasmic anchor protein allows translocation of the transcription factor into the nucleus. One example of phosphorylation-regulated nuclear translocation is provided by the Rel family of transcription factors which includes NF-κB (Hunter and Karin, 1992). NF-κB is activated in many different cell types following a stimuli. In unstimulated cells, the majority of the NF-κB p50/p65 proteins is retained in the cytoplasm, through binding to IκB inhibitor proteins. IκB proteins mask the DNA-binding activity of NF-κB and control the nuclear uptake of associated DNA binding proteins. Following stimulation with TPA, the NF-κB complex translocates to the nucleus in an active form. External stimuli activate several protein kinases such as PKA or PKC which can induce the phosphorylation of IκB. This phosphorylation leads to dissociation of IκB from the NF-κB complex and to the unmasking of the DNA-binding domain. This is followed by translocation to the nucleus where NF-κB binds to κB sites to activate transcription of target genes.

The DNA binding activity of nuclear transcription factors can be modulated by phosphorylation either positively or negatively (Lüscher et al., 1990). One example of negative regulation by phosphorylation is the c-myb protein. Phosphorylation of c-myb by casein kinase II (CKII) results in a large decrease in DNA binding activity. Another example

is c-Jun. In non-stimulated cells, c-Jun is present in a form that is phosphorylated on sites that inhibit DNA binding activity. In response to cell stimulation with growth factors, c-Jun undergoes dephosphorylation of these sites and its DNA binding activity increases (Boyle et al., 1991).

Phosphorylation can affect the interaction of transcription factor transactivation domains. Transcription factors are usually composed of separable DNA-binding and transcriptional activation domains. In most cases, phosphorylation effects on transactivation are of positive control. As shown earlier, PKA regulates CREB activity by phosphorylation of its activation domain at Ser-133. c-Myc is an example of a transcription factor whose transactivation function is stimulated by phosphorylation. Ser-62 is a MAP/ERK kinase phosphorylation site in human c-Myc and is stimulated by growth factors treatment.

*Regulation of transcription initiation by RNA polymerase II (pol II)*

Regulation of transcription initiation is by far the most widespread form of gene control in eukaryotes (Hernandez, 1993). Such control results in the response to extracellular signals, leading to changes in levels of specific mRNAs and their translation products. In eukaryotes, initiation is governed by DNA sequence elements comprising several functional classes. Core promoter elements contain binding sites for general transcription factors leading to recruitment of RNA pol II and control the location of transcription initiation sites. Upstream promoter elements and enhancers regulate the rate at which RNA pol II initiates new rounds of transcription from the core promoter. Seven general transcription factors have

been identified and extensively purified. Among these factors, only TFIID contains a DNA binding activity with specificity for the TATA box. The others and RNA pol II enter the transcription complex by protein-protein interactions. The largest subunit of RNA pol II contains a C-terminal domain (CTD). Hyperphosphorylation of the CTD blocks incorporation of pol II into the initiation complex.

Initiation of transcription by RNA pol II requires the formation of a complex containing the basal factors TFIID, TFIIA, TFIIB, TFII E/F, TFIIH, TFIIJ and RNA pol II (Buratowski, 1994) (Fig. 1.2.2). The first step in the assembly of the initiation complex is binding of TFIID (the TATA box binding protein (TBP) and associated factors) to the TATA element. It provides a recognition site for the association of the other general transcription factors and pol II. TFIIA promotes stable binding of TFIID to the core promoter and has been shown to bind stably to TATA box binding protein (TBP). TFIIB binds directly to the TFIID-DNA complex and serves as a bridge for the recruitment of RNA pol II linked to IIF into the initiation complex. However, with purified factors TFIIA appears not essential for basal transcription. It does function to establish and maintain the committed complex under more physiological conditions.

The formation of the DB-polF or DAB-polF complex leads to the association of the remaining factors (TFIIE, TFIIH, and TFIIJ) required for basal transcription. The function of TFIIE is not clear, but TFIIE incorporation appears necessary for subsequent recruitment of TFIIH. TFIIH possesses a kinase activity that is capable of phophorylating the CTD of pol II. The CTD is in a non-phosphorylated (Pol IIA) form in the preinitiation complex

where it is engaged in an interaction with TFIID. RNA pol IIO is the phosphorylated form found in the elongation complex. Thus, each transcription cycle may be associated with the reversible phosphorylation of the CTD. The subsequent phosphorylation of the CTD is thought to be a key step in the progression from an initiation complex to an elongation complex. TBP interacts only with the nonphosphorylated form of RNA pol II which is the form known to enter the initiation complex. In addition to interacting with the general transcription factors, TBP interacts with several factors termed negative cofactors (NC)1, NC2, and Dr1. These factors have a negative effect on core promoter function and interfere with the binding of TFIIA. Thus, phosphorylated Dr1 can associate stably with TBP on a TATA box and displace TFIIA. Transcription of protein-coding genes can be stimulated by transcriptional activators, sequence-specific transcription factors such as stimulated protein 1 (SP1), upstream stimulator factor (USF), AP-1 and CREB. As stated earlier, these factors contain two principal domains (1) a sequence-specific DNA-binding domain and (2) an activation domain which allow them to stimulate transcription synergistically in combination with other activators. Transcription initiation is thought to be one of the targets of these regulatory proteins.

*Regulation of mRNA stability*

The control of mRNA stability is important in determining the levels of gene expression during cell growth (Ross, 1995; Beelman and Parker, 1995). Recent studies have defined several mechanisms of regulation of RNA stability.

The general mRNA decay pathway is initiated by shortening of the poly (A) tail followed by decapping and 5' to 3' exonucleolytic degradation of the transcripts. Messenger RNAs lacking the cap structure are rapidly degraded in many eukaryotic cells. A variation of this pathway has been observed in which transcripts undergo 3' to 5' decay after poly (A) shortening. Messenger RNA decay can also be initiated by decapping followed by 5' to 3' decay of the transcript independent of poly (A). Degradation of mRNAs containing nonsense codons is part of a process termed mRNA surveillance, that ensures the rapid degradation of aberrant transcripts.

Another possible pathway leads to decay of unstable mRNAs through a 3'-AU-rich instability determinant binding some cytoplasmic factors (Ross, 1996). Many transcription factors (c-fos, c-myc) and cytokines as well contain such elements regulated by different signal transduction pathways. The AU-rich elements (ARE)s and their associated proteins interact with poly (A)-binding protein (PABP), forming a bridge between the ARE and the poly (A) tail. It has been shown that PABP in association with the poly (A) tail protects the 3' end of mRNAs from nuclease attack. An association between PABP and the ARE might allow PABP to be released from the poly (A) tail. The poly (A) tail might then be susceptible to complete degradation. It is suggested that the AU-rich sequences may play a role in nuclear export or nuclear mRNA decay as well as in cytoplasmic mRNA decay. A family of ARE-binding proteins (AUBPs) has been described. Some are primarily cytoplasmic, while others are nuclear or shuttle between both compartments. Several observations suggest that AUBPs influence mRNA stability (Ross, 1996). AUBP abundance, or activity, increases

or decreases as mRNA decay rates change.

Some unstable mRNAs such as Fos and myc mRNAs contain a coding region instability determinant (CRID) (Schiavi et al., 1992). Ribosomes may carry associated factors involved in mRNA decay. The movement of ribosomes across the CRID sequence may alter the ribosome and /or associated factors. This alteration may lead to rapid and complete digestion of the poly (A) tail. Once the poly (A) tail is removed, the transcribed portion of the mRNA is rapidly degraded.

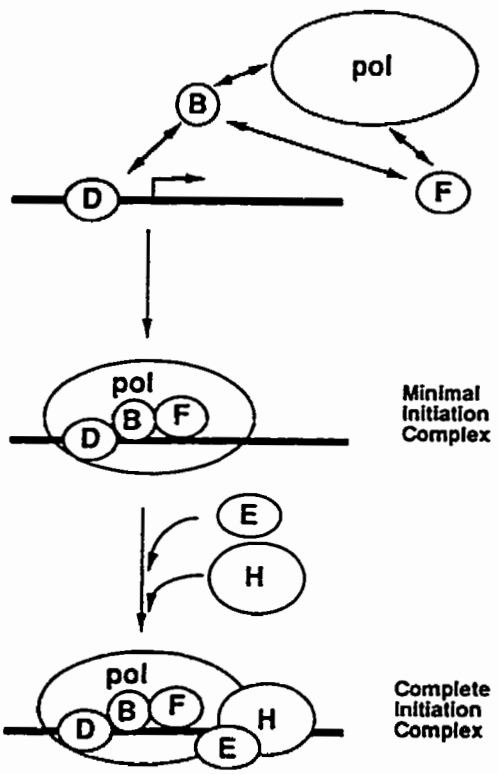


Fig.1.2.2 Schematic model of transcription initiation complex assembly (From Buratowski, S. Cell, 77: 1-3, 1994).

## PART B: GENERAL INTRODUCTION TO THE PROJECT

### 1.4 GENE EXPRESSION AND BIOLOGICAL ACTION OF INSULIN-LIKE GROWTH FACTORS (IGFS), EPIDERMAL GROWTH FACTOR (EGF) AND BASIC FIBROBLAST GROWTH FACTOR (bFGF) DURING DEVELOPMENT

All cells in the body are derived from a fertilized egg. In order to reproduce and multiply, every cell must undergo an orderly series of events, generally called the cell cycle (Fig. 1.4.1). The full mature cell is said to be in Go phase (G stands for gap). At this stage, the cell is not committed to division. G is the decision phase in which cells either commit to undergo another round of DNA synthesis (S phase) and continue to cycle, or to exit the cell cycle to enter a quiescent stage, referred to as Go phase. When DNA synthesis is completed, cell normally proceed to G2 which is a short period where some protein synthesis occurs. During this time, cells prepare to enter into mitosis (M phase).

Control of cell proliferation is a result of coordinated regulation of multiple biochemical pathways that integrate both intracellular and extracellular signals. A number of hormones are essential to normal body growth such as somatotropin and insulin. During the last decade, it was found that some growth factors, such as insulin-like growth factor I and II (IGF-I and II), epidermal growth factor (EGF), and fibroblast growth factor (FGF) are mitogenic polypeptides. Although the ontogeny and targets of IGF, EGF and bFGF actions are largely unknown, these growth factors are considered important for cellular differentiation and growth.

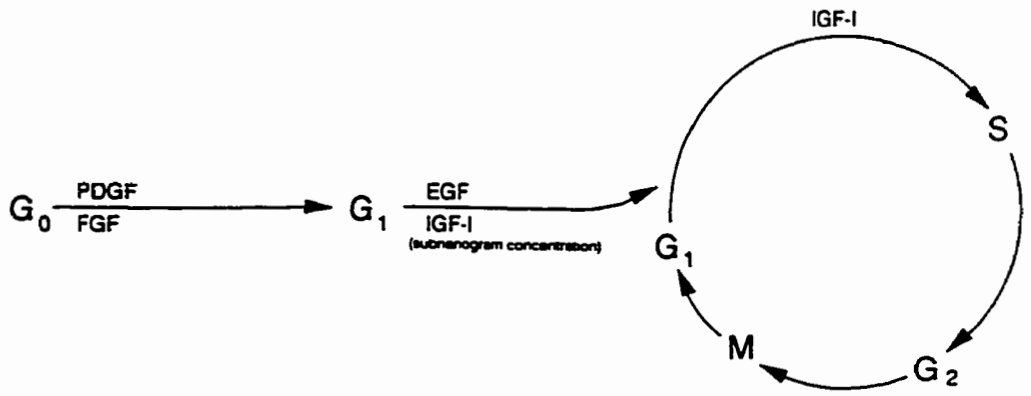


Fig. 1.4.1 Illustration of some growth factors involved in the cell cycle at different phases (From Lowe, W. L. Jr. In: Insulin-like growth factors: Molecular and cellular aspects, pp. 49-85).

1.4.1 Insulin-like growth factor I and II (IGF-I and -II)

IGF-I and IGF-II are non glycosylated single-chain peptides with three intrachain disulfide bridges consisting of 70 (7.6kDa) and 67 (7.5kDa) amino acids, respectively (Daughaday and Rotwein, 1989). The mature molecule of IGF-I contains four domains, an N-terminal B region followed by C, A and D domains (Foyt and Roberts, 1991). The B, C and A domains are structural homolog of proinsulin. The IGF-I prohormone has an additional E domain which varies in length due to alternative splicing of the primary transcripts. A single gene encodes human IGF-I which consists of 6 exons distributed over

nearly 70 kb of chromosomal DNA (Kim et al., 1991; Fig. 1.4.1.1). Several transcription start sites were identified within IGF-I exons 1 and 2, adjacent to distinct promoters upstream of exons 1 and 2, respectively, and at least three polyadenylation sites were mapped to exon 6. Genomic DNA encoding the 5' region of the porcine IGF-I gene was cloned and sequenced and shown to be highly homologous to that of man, rats and sheep (Weller et al., 1993). The primary transcript of the IGF-I gene can be alternatively spliced to result in two mRNAs encoding either precursor proteins IGF-Ia or IGF-Ib differing in their carboxy-terminal amino acid extensions. Although the biological activities generated from these alternative precursors have yet to be determined, alternative RNA splicing may provide a mechanism for regulating IGF-I biosynthesis and subsequent function. A 40 kb single gene encoding human IGF-II contains 9 exons which are transcribed from four promoters. In addition there are two polyadenylation sites (Sussenbach et al., 1991; Fig. 1.4.1.2). The mature IGF-II polypeptide is encoded by exons 7, 8 and part of 9. Promoter 1 (P1) is thought mainly expressed in adult liver required for transcription of leader exons 1, 2 and 3, whereas homologue of P1 is absent in the rat and mouse gene (Ikejiri et al. 1990; Rotwein and Hall, 1990). Therefore, in contrast to the rodent liver, in which IGF-II expressed is repressed to zero after birth, expression of IGF-II in human liver persists postnatally. Initiation of transcription at these various sites and alternative RNA splicing can generate alternate mRNA species. For both IGFs genes, the use of different promoters contributes at least in part, to the observed developmental and tissue specific patterns of IGFs gene expression.

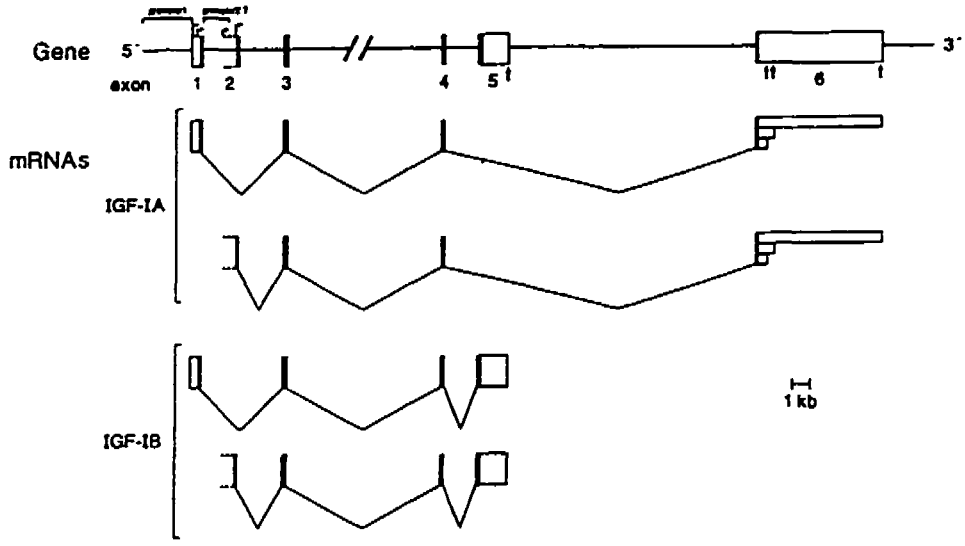


Fig. 1.4.1.1 Structure and expression of the human IGF-I gene. An overview of the human IGF-I gene organization and its different mRNAs is presented. Exons 1-6 are indicated by boxes, with coding regions in black and noncoding regions in white. Promoter 1 and putative promoter 2 are labelled on the gene. Not all transcription start sites have been drawn on the map. Since the 5'-end of exon 2 has not been established with certainty, it has been left open. Sites of pre-mRNA processing are indicated by thin lines. Polyadenylation sites are marked by vertical arrows in the gene and by box of varying length at the 3'-end of the mRNAs (From Kim, S.W., Rosemarie, L and Rotwein, P., Mol. Endocronl., 5: 1964-1972, 1991).

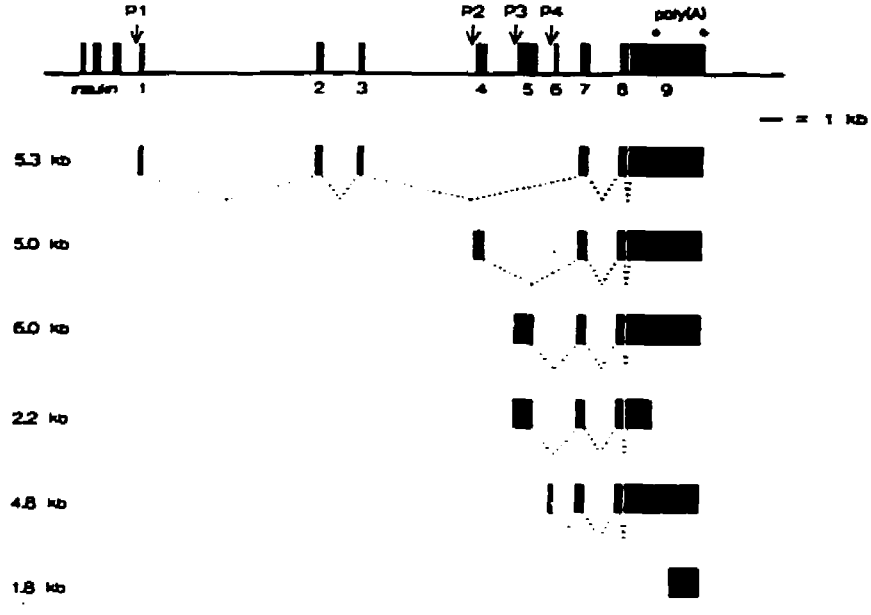


Fig. 1.4.1.2 Structure of the human IGF-II gene organization and mRNAs species. The gene consists of 9 numbered exons, 4 promoter sites indicated by the arrows (P1-4). The asterisks show two alternative polyadenylation sites. Initiation of transcription at P1, which is mainly active in adult liver, generates an IGF-II mRNA of 5.3 kb. The mature IGF-II polypeptide is encoded by exons 7, 8 and part of 9 (From Sussenbach, J.S., Steenbergh, P.H., Hansen, E., Meinsma, D., van Dijk, M.A., Holthuizen, P., de Moor, C.H., Jansen. M. and Van den Brande, J.L. In: Modern Concepts of Insulin-like Growth Factors, pp639-654, 1991).

*IGFs during development*

IGF-I and -II are potent mitogenic growth factors which are capable of inducing cell proliferation and differentiation (Lowe, 1991). Transgenic mice for IGF-I exhibited an increased level of IGF-I with an increase body weight (Mathrew et al., 1988). Conversely, embryonic mice with inactive IGF-I gene were significantly smaller than their normal litters (Powell-Braxton et al., 1993). They have severe muscle dystrophy and the majority of these mice die at birth. Mice lacking a functional IGF-II gene were viable but are 60 % of the size of wild-type litter mates (de Chiara et al., 1990). These observations suggest an important role for IGF-I and -II in promoting both pre- and postnatal growth.

Developmental and tissue specific pattern of IGFs expression has been characterized with the rat model (Hogg et al., 1994; Brown et al., 1986; Rotwein et al., 1987). Both IGF-I and -II mRNAs were detectable in rat embryos as early as 11 days (Rotwein et al., 1987). Over the gestation period, IGF-II transcripts were more abundant than IGF-I. Brown et al. (1986) found that IGF-II mRNAs were present in multiple tissues such as liver, kidney and muscle from the fetal to postnatal period. In the fetal rat, IGF-II mRNA was found to be abundant in most tissues including liver, intestine, lung and brain but decreased markedly at birth in all tissues except in the brain (Lund et al., 1986). During postnatal life, a decrease in IGF-II mRNA levels corresponded to a decrease in circulation levels of IGF-II (Moses et al., 1980). In the rat, IGF-I mRNA was higher in most fetal tissues, with the exception of liver where IGF-I mRNA level was higher in the adult (Lund et al., 1986), paralleling an increase in plasma IGF-I level (Donovan et al., 1989). Han et al. (1988) found that IGF-I

and II mRNAs were present in human fetal tissues such as pancreas, liver, kidney and muscle at 16 to 20 weeks of gestation. The same authors reported that IGF-II mRNA was present in greater quantities than that of IGF-I in most tissues during early fetal life. Similar to rat, human adult liver expressed higher IGF-I mRNA than fetal liver (Han et al., 1988). In ovine, the level of IGF-II mRNA was high in various tissues from early gestation and decreased with maturation (O'Mahoney et al., 1991). In the pig, IGFs mRNA levels were also detected in multiple tissues during the fetal life (Lee et al., 1993). Messenger RNA for IGF-I was most abundant in the skeletal muscle and least abundant in the liver, showing that expression of IGF-I and -II are tissue and developmentally regulated.

IGFs are released from many cell lines in culture and synthesized in various tissues (Rutanen and Pekonen, 1990). Tissue IGF-I has been detected in the rat (D'Ercole et al., 1980), the human (D'Ercole et al., 1986) and the pig (Hausman et al., 1991). Multiple tissues express IGF-I peptide including liver, kidney and muscle, suggesting an autocrine and /or paracrine role of IGF-I during development. Changes with age in circulating concentrations of IGFs have been characterized in humans (Ashton et al., 1985; Bala et al., 1981) rats (Donovan et al., 1989) and pigs (Lee et al. 1991). In humans and rats, IGF-I serum concentrations are low around birth, but rise postnatally (Ashton et al., 1985; Bala et al., 1981; Donovan et al., 1989). Concentration of IGF-II in rat was high prenatally and declined rapidly after birth (Moses et al., 1980), whereas, in humans, it was low prenatally and increased after birth (Enberg and Hall, 1984). Serum IGFs pattern in the pig is similar to human but different from that in the rat. In the pig, IGF-II concentration was higher than

IGF-I and both increased during development (Lee et al., 1991). All these findings suggest that IGFs produced by the tissues and present in the serum act as important regulators of animal growth and development.

*IGF receptors during development*

The biological effects of IGFs are mediated through binding to high affinity receptors on the cell surface (Werner et al., 1991). Targeted disruption of the IGF-I receptor gene resulted in growth-retarded mouse embryos (Liu et al., 1993). In fact, growth was retarded to about 70 % of that seen in normal mouse embryos, strongly suggesting that the IGF-I receptor plays a major role in embryonic growth. Receptors of IGF-I are distributed in the majority of tissues and cells (Rechler and Nissley, 1985). Two types of IGF receptors, based on their relative affinities for IGF-I, IGF-II and insulin, have been identified (Werner et al., 1991; Nissley et al., 1991). The type I receptor, like the insulin receptor, is a glycoprotein with a molecular weight between 300 kDa and 350 kDa which consists of two extracellular $\alpha$-subunits ($\approx$130 kDa) and two $\beta$-subunits ($\approx$95 kDa) containing a transmembrane and a tyrosine kinase domain (Werner et al., 1991). In the mature receptor, two $\alpha$ and $\beta$ chains are joined by disulfide bonds. The type I receptor shows both structural and functional similarities with the insulin receptor and has high affinity for IGF-I and IGF-II, but a much lower affinity for insulin. The signal transduction is thought to be mediated by activation of the tyrosine kinase domain. By contrast, the type II receptor is a monomeric, transmembrane 250 kDa protein with a short cytoplasmic domain that lacks kinase activity (Nissley et al., 1991). The type II receptor has no structural homology with insulin or type I receptor. It is

identical to the cation-independent mannose-6-phosphate receptor which directs mannose-6-phosphate-containing proteins to lysosomes. IGF-II binds with high affinity to type II receptor while IGF-I binds less avidly, and insulin does not bind at all to the type II receptor. It has become increasingly clear that type I receptor mediates most of the well known biological effects of both IGF-I and -II. The role of type II receptor in IGF-I or -II-mediated transmembrane signalling is still obscure. In addition to the above receptors, another receptor named IGFII-XR has been tentatively identified from placenta. Lack of this receptor results in reduced placental growth (Baker et al., 1993).

In the rat, levels of IGF-I and -II receptor mRNAs were determined in various tissues from late fetal to early postnatal development (Werner et al., 1989; Sklar et al., 1992). The IGF-I and -II receptor mRNA levels were high in multiple tissues of fetuses and decreased to much lower levels during postnatal life (Werner et al., 1989; Sklar et al., 1992). The IGF-II receptor is present and expressed in a variety of human tissues including pancreas, liver and stomach (Funk et al., 1992). The highest amount of IGF-II receptor was detected in kidney, heart and thymus and the lowest receptor content was measured in muscle and brain. In the pig, it was found that at day 20 of pregnancy, IGF-I receptors were present in the placenta, but only in endometrial cells (Chastant et al., 1994). In contrast, IGF-II receptors were detected on whole embryos from day 8 of pregnancy (Chastant et al., 1994). The variable amounts of IGF receptors present in different tissues make them likely candidates for regulatory processes that might be involved in organ and tissue growth and development.

*IGF binding proteins during development*

In the circulation and body fluids, IGFs are bound to specific proteins which are synthesized in the liver and in other tissues (Clemmons, 1991). To date, six forms of IGF binding proteins (IGFBPs) that range in size from 24-45 kDa have been identified. Their cDNAs have been cloned and designated IGFBP-1 to -6 (Shimasaki and Ling, 1991). The predominant binding protein in serum of adult rat and human is IGFBP-3, which binds 95 % of endogenous IGF-I (Jones and Clemmons, 1995). IGFBP-1 has a similar affinity for IGF-I and IGF-II, while IGFBP-2 and IGFBP-3 have greater affinity for IGF-II and IGF-I respectively. The function of these proteins is not completely understood. It is hypothesized that the IGFBPs (1) act as transport proteins in plasma to control the efflux of IGFs from the vascular space, (2) serve as reservoir for IGFs prolonging the biological half-life of the IGFs (3) provide a means of tissue- and cell type-specific localization (McCusker and Clemmons, 1992). In the extracellular fluids, IGFBPs can alter the IGF-I and II binding capacity to receptors on the surface of intact cells (Jones and Clemmons, 1995). It has also been suggested that the IGFBPs function as major modulators of IGF action either as inhibitors (Frost et al., 1993) or enhancers (Conover, 1992). Transgenic mice overexpressing human IGFBP-3 resulted in a liver, spleen and heart significantly heavier than that of normal mice (Murphy et al., 1995). Transgenic mice that overexpressed rat IGFBP-1 had a significant reduction of 83-92% of the body weight (Rajkumar et al., 1995). The authors also found that the difference in body weight between transgenic and wild-type mice was most apparent around the time of weaning when the transgenic mice showed a more marked growth

retardation. In addition, the transgene was highly expressed in brain, uterus, lung, kidney and heart, but little expression was detected in the liver, thus showing that IGFBPs may function to inhibit IGFs action *in vivo* and this inhibition selectively impairs development of specific organs.

Messenger RNAs of IGFBPs and their proteins were detected in various tissues during development (Clemmons, 1991). During the postnatal life in rat, IGFBP-3 mRNA was expressed in a large number of tissues (Albiston and Herington, 1992). The kidney had the greatest amount of IGFBP-3 mRNA with moderate mRNA levels in liver, stomach and placenta. IGFBP-1 mRNA was present in many human fetal tissues, such as liver, gut, kidney and lung (Hill et al., 1990). Like IGFs, IGFBPs are also subject to different developmental and tissue-specific regulation. In rat, levels of both IGFBP-1 and -2 mRNAs were the highest in liver, with abundant IGFBP-2 mRNA present in fetal kidney, stomach and brain (Ooi et al., 1990). Similar to rat, IGFBP-2 mRNA levels in ovine tissues were high in early gestation and decreased with maturation thus following the same pattern of expression as IGF-II (Delhanty and Han, 1993). Multiple isolated human fetal tissues release IGFBPs *in vitro* including fibroblasts and myoblasts (Hill et al., 1989), liver explants (Lewitt and Baxter, 1989), and pancreas explants (Hill et al., 1987). The immunopositive staining for IGFBP-1, -2 and -3 was found in many human fetal tissues, being prominent in kidney, pancreatic endocrine tissue and skeletal muscle fibres (Hill and Clemmons, 1992), supporting the idea that IGFBPs might exert differential effects in various tissues during development and growth. In the pig, IGFBP-2 was more abundant in fetal than in postnatal serum. During

postnatal life, the liver IGFBP-3 mRNA expression and serum IGFBP-3 concentrations increased with serum IGF-I levels (Lee et al., 1991; Kampman et al., 1994). These data suggest that mRNA levels as well as tissue and serum concentrations of IGFBPs are influenced by age. The IGFBPs are present in multiple tissues and serum, implying that IGFBPs are important in regulating IGFs actions.

*Biological action of IGF-I and IGF-II*

The two main biological actions of the IGFs may be summarized as insulin-like metabolic and growth promoting actions. With the use of primary cell culture or organ explants, it has been demonstrated that both IGF-I and IGF-II stimulate glucose uptake, glycogen and lipid synthesis in adipose tissue and stimulate cell proliferation and differentiation (Froesch et al., 1985; Lowe et al., 1991). Anabolic effect of IGF-I in rats was reported by Tomas et al. (1993) who observed that administration of IGF-I over a 14 day period to growing rats led to an increased body weight gain, an improved nitrogen retention and a greater food conversion efficiency. IGF-I is though to mediate many of the physiological actions of GH on somatic growth (Copeland et al., 1980). Transgenic mice expressing GH exhibited increased whole body growth rate after IGF-I gene expression was induced (Mathrew et al., 1986). Targeted mutagenesis of the genes encoding IGF-I and -II demonstrated the importance of IGFs in the growth process (Baker et al., 1993). The authors conclude that IGF-I, a previously known mediator of postnatal GH action, is also involved in embryonic development. The essential growth-promoting function of IGF-II is believed

to be restricted to the period of embryogenesis in the mouse (de Chiara et al., 1990). However, Wolf et al. (1994) found that postnatally elevated IGF-II in transgenic mice displayed increased kidney, testicles and adrenal weight at the age of 4 to 12 weeks although body growth was not significantly changed.

*IGFs and pancreas* Specific receptors for IGF-I have been detected on rat pancreatic cells and murine acinar tissues (Williams et al., 1984; Miettinen et al., 1993). Messenger RNAs of IGFs and IGFBPs were detected in rat pancreas (Hogg et al., 1994). Other studies have shown that cultured fetal and neonatal rat islets and human fetal pancreatic explants release immunoreactive IGF-I (Scharfmann et al., 1989; Romanus et al., 1985; Hill et al., 1987) and IGF-II (Bryson et al., 1989) into the medium. By immunohistochemical localization, IGF-I was reported in A cells whereas IGF-II has been observed exclusively in B-cells in man, dog and rat (Maake and Reinecke, 1993). Recently, Hayakawa et al. (1996) reported that following 95% pancreatectomy, gene expression of IGF-I and IGF-I peptide level was rapidly induced in the remnant pancreas. The authors suggested that the newly synthesized IGF-I may stimulate ornithine decarboxylase (ODC), which is an early event in cell proliferation, inducing DNA replication and tissue regeneration. These observations suggest that IGFs may be involved in pancreatic growth and development.

*IGFs and liver* The liver is believed to be the main producer of circulating IGF-I in adult rats and human (Daughaday and Rotwein, 1989; Scharf et al., 1995). It is also an important

source of circulating IGFBPs in the rat (Daughaday and Rotwein, 1989). Both IGF-I and -II are expressed in liver of rat (Zarrilli et al., 1992; Streck and Pintar, 1992) and pig (Lee et al., 1993). It has also been observed that IGF-I receptors are present in the rat liver (Santos et al., 1994). During development, binding of IGF-I to liver membranes is 4.5 times higher in 20 day-old fetuses than in adult rats (Santos et al., 1994). It has been reported that IGFs are involved in liver regeneration (Caro et al., 1988; Burguera et al., 1990). In fact, the number of IGF-I, IGF-II/M-6-P receptors and the level of IGF-II receptor mRNAs are greater in regenerating hepatocytes than in normal liver cells. The [$^{125}$I] IGF-II binding to crude plasma membranes from regenerating liver was maximal 2 days after hepatectomy. This increase in binding in regenerating liver was associated with an increase in the concentration of IGF-II receptors (Burguera et al., 1990). It was also observed that gene encoding rat IGF-I and IGFBP-1 were induced in regenerating rat liver (Mohn et al., 1991). Thus, IGF-I and IGFBP-1 may interact with hepatocytes to promote liver regeneration. These studies also suggest that IGFs are important stimulators of liver growth and regeneration.

*IGFs and kidney* Several lines of evidence suggest that IGF-I can affect kidney growth and renal function. Overexpression of IGF-I in transgenic mice induced kidney growth (Mathrew et al., 1988). During development, high levels of IGFs and IGFBPs mRNAs have been detected in fetal kidney of human (Han et al., 1988; Suikkari et al., 1992) and rodent (Stylianopoulou et al., 1988; Price et al., 1995). In rat, the renal IGF-II mRNA levels rapidly decreased after birth (Stylianopoulou et al., 1988) whereas the IGF-I mRNA

increased after birth (Albiston and Herington, 1992). It was observed that the collecting duct of the kidney is a major source of IGF-I which is also produced by glomerular mesangial cells in culture (Bortz et al., 1988; Aron et al., 1989). Both IGFBPs and IGF-I are synthesized in the kidney (Aron et al., 1991). It has been shown that IGF-II receptors are present in glomeruli and in both basolateral and brush border membranes of proximal tubular cells (Haskell et al., 1988). IGF-I mRNA level and peptide concentration as well as IGF-I binding increased in the generative zone of the post-ischemic regenerating rat kidney (Matejka and Jennische, 1992). In young male rat, unilateral nephrectomy resulted in a compensatory growth of the remaining kidney (Fagin and Melmed, 1987), where IGF-I mRNA level increased within 24 h, and remained at high level for at least 7 days. Levels of IGF-I and -II receptors and their mRNAs in whole kidney were also increased (Mulroney et al., 1992), suggesting that IGF-I has a trophic effect during kidney regeneration.

*IGFs and skeletal muscle* In the skeletal muscle, both IGF-I and -II exhibit a range of physiological effects that includes glucose uptake (Bevan et al., 1992), protein metabolism (Fryburg, 1994), acceleration of the rate of DNA synthesis, and enhancement of myoblast differentiation (Rosen et al., 1993). Transgenic mice overexpressing IGF-I have myofiber hypertrophy (Coleman et al., 1995). Messenger RNA for IGF-II has been detected in muscle of 12 day-old rat fetuses (Stylianopoulou et al., 1988). In the rat, mRNA of IGF-II receptor was in great abundance in fetal muscle and decreased after birth, but it was still more abundant than that of IGF-I in 4 week-old rats (Alexandrides et al., 1989). The number of

receptors for IGF-I is constant from birth to 4 weeks of age in the rat (Alexandrides et al., 1989). Using L6A rat myoblasts incubated with IGF-II, Bach et al. (1995) have demonstrated that IGF-II has a primary role in promoting the differentiation and growth of skeletal muscle predominatly through the IGF-I receptor (Bach et al., 1995). In addition, IGFs are believed to be involved in muscle regeneration and growth (Levinovitz et al., 1992) since during the regeneration process, levels of both IGF-I and -II mRNA as well as IGFs proteins were transiently induced. This suggests that tissue IGFs could act locally to stimulate tissue growth and /or differentiation.

1.4.2 Epidermal growth factor (EGF)

Epidermal growth factor is a 53 amino acid polypeptide with three disulfide bonds. It is a powerful stimulator of proliferation and differentiation of a variety of cell types *in vitro* as well as *in vivo* (Fisher and Lakshmanan, 1990). The EGF is initially synthesized as a large (130 kDa) precursor molecule with the mature EGF sequence located near the C-terminus (Carpenter and Wahl, 1990). The sequence shows two hydrophobic domains, one representing a signal sequence and the other a transmembrane domain, indicating that the precursor is a transmembrane protein. The cytoplasmic domain of the precursor has no known function. The extracellular domain of the EGF precursor contains, in addition to the sequence for mature EGF, eight other EGF-like domains. The uncleaved precursor is capable of binding to the EGF receptor and shows biological activity. Thus, pro-EGF functions not only as a source for soluble EGF, but may also have a role in mediation of intercellular

communication between cells with pro-EGF on their surfaces and cells with EGF receptors (Carpenter and Wahl, 1990). Proteins of the EGF family act via a surface EGF receptor (EGFR) which is an approximately 170 kDa (1186 amino acids) transmembrane glycoprotein encoded by the c-erbB proto-oncogene (Velu, 1990). The EGF receptor consists of an extracellular domain with a high cysteine and N-linked glycosylation content, one transmembrane domain, and a cytoplasmic domain with tyrosine kinase activity. Targeted disruption of EGFR gene in mice reduced life span and resulted in abnormalities in skin, kidney, brain, liver and gastrointestinal tract (Threadgill et al., 1995), indicating that the receptor in involved in a wide variety of cells.

*EGFR during fetal development*

It has been shown that EGFR mRNA and protein are expressed from the very early stage of the mouse embryo (Wiley et al., 1992). Adamson and Meek (1984) have studied the ontogeny of the EGF receptor in several tissues of the fetal mouse, and observed its presence in the whole embryos and placenta with different patterns of expression among tissues (Fig.1.4.2.1). In fetal bovine, immunocytochemical studies revealed that EGFR was localized in the ductal and tubular epithelium of mesonephros and associated organs of the urogenital tract (Winters et al., 1993). In addition, in bovine mesonephric cells, EGF induced DNA synthesis and tyrosine phosphorylation, suggesting that EGFR detected in these cells was functional. Messenger RNA of EGF receptor was present in human fetal ovary and uterus during the first- and second-trimester of gestation (Yeh et al., 1994). Receptors for EGF

have also been found in human fetal kidney, liver and lung (Nexø et al., 1989) and sheep placenta (Lacroix and Kann, 1993). In the pig, mRNAs for EGF and EGFR were expressed in the conceptus at all stages of development, suggesting that EGF could be involved in regulating growth and development of early pig conceptus (Zhang et al., 1992). Thorburn et al. (1981) observed that infusion of recombinant EGF to ovine fetus for 3-14 days produced skin hypertrophy and increased liver, kidney, adrenal and thyroid weights. It has also been reported that the development of mouse embryos is improved by the addition of EGF in culture (Kuo et al., 1990). Taken together, all these studies suggest that EGF acts as a regulator of mammalian embryonic and fetal development.

*EGF during development*

It is thought that EGF plays pivotal roles as an autocrine and / or paracrine regulatory signal during development. Messenger RNAs of EGF and pro-EGF were identified in most adult rodent tissues. Levels of mRNA were the highest in the salivary gland and the kidney (Rall et al., 1985). In human, mRNA of EGF was expressed in the kidney, the salivary and the thyroid (Kajikawa et al., 1991). A wide range of pig tissues expressed EGF mRNA (Vaughan et al., 1991). The highest level of EGF mRNA was found in kidney and pancreas, with lower levels in submaxillary gland and seminal vesicle. EGF is present in multiple body fluids such as plasma, salivary, pancreatic and gastric fluids (Capenter and Wahl, 1990). The authors found that the concentration of EGF in tissue is generally low. Tissues with high level of pro-EGF mRNA and EGF protein are salivary gland, kidney, mammary gland and

pancreas. During the early neonatal period, EGF levels are low in most mouse tissues (Perheentupa et al., 1985; Laborde et al., 1988). Tissue concentration of EGF was shown to increase in the mouse during the first 2 months of postnatal life. Vaughan et al. (1992) reported that EGF was present in extracts of kidney and pancreas, as well as in urine, but was not detected in extracts of submaxillary gland or liver of the pig. Thus, many tissues appear to synthesize EGF indicating that EGF may be involved in various organs growth and development.

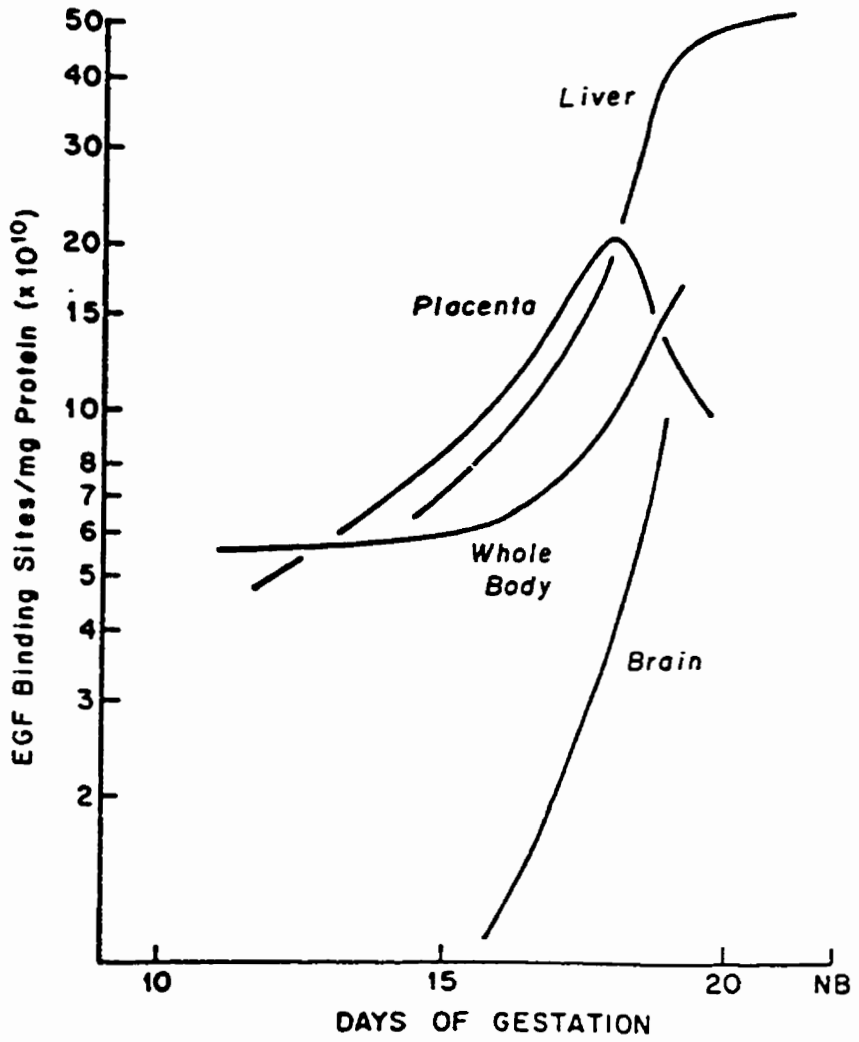


Fig.1.4.2.1 Ontogeny of EGF receptors in the fetal mouse (From Fisher, D.A. and Lakshmanan. Endocr. Rev., 11: 418-442, 1990).

*Biological action of EGF*

The best documented activity of EGF is its ability to induce DNA synthesis and to promote proliferation and differentiation of mesenchymal and epithelial cells (Carpenter and Wahl, 1990).

It has been reported that EGF stimulates gastrointestinal, liver, and pancreatic maturation (Marti et al., 1989; Logsdon, 1986). In addition, EGF has shown therapeutic potential as an accelerator of wound healing (Schultz et al., 1990). Recently, it was found that EGF exerted mitostimulatory action and stimulated protein synthesis in fetal bovine myoblasts (Blachowski et al., 1993). There is evidence that EGF is involved in animal growth and development since daily administration of EGF antiserum to neonatal mice reduced weight gain by 40% during the first postnatal 4 weeks (Zschiesche, 1989).

*EGF and pancreas* Several studies suggest a role for EGF in the growth and exocrine function of the pancreas. Relatively high levels of EGF (Hirata and Orth, 1979; Hirata et al., 1982) and EGFR (Hormi and Lehy, 1994) have been observed in human pancreatic tissue and juice. Korc et al. (1983) reported that pancreatic acinar cells express the EGF receptor. It was observed that EGF stimulates pancreatic acinar cell growth, increases the rate of DNA and protein synthesis and amylase release in cultured pancreatic acini (Logsdon, 1986; Brannon et al., 1988; Logsdon and Williams, 1983). Intraperitoneal administration of EGF in rats stimulated pancreatic DNA synthesis and increased pancreatic gland weight; the effect was similar to the maximal effect obtained with cholecystokinin (CCK) and secretin (Dimbinski et al., 1982).

*EGF and liver* Administration of EGF to neonatal rabbit increased liver weight, DNA and protein concentrations, bile flow and bile salt excretion (Opleta et al., 1987). Ishigami (1993) reported that in primary cultured hepatocytes, EGF stimulated DNA synthesis after 12 h and reached a peak at 48 h. The level of EGF receptor mRNA significantly increased above control level after 24 and 72 h of partial hepatectomy (Johnson et al., 1988). In addition, normal mice showed a rapid increase in plasma EGF levels 8 h after partial hepatectomy (Noguchi et al., 1990), suggesting a possible role of EGF in liver regeneration.

*EGF and kidney* In human, EGF was first purified from urine (Gregory, 1975). It was found that levels of EGF in urine were much higher than that in blood (Fisher et al., 1989), suggesting that in human, kidney is the source of urinary EGF. In fact, EGF was detected in human distal nephron (Nouwen and de Broe, 1994). Administration of EGF resulted in a number of renal responses, including decreased renal blood flow and glomerular filtration rate, increased urine flow, inhibition of $Na^+$ reabsorption, and altered tubular transport function (Harris, 1991). The growth and differentiation of renal proximal tubular cells *in vivo* is under the control of a number of growth factors, including EGF (Hammerman et al., 1993). *In vitro*, EGF has been shown to regulate renal metabolism, filtration and transport processes (Harris and Daniel, 1989; Vehaskari et al., 1989). Exogenous EGF administration accelerated renal tubular regeneration following gentamicin induced nephrotoxicity (Morin et al., 1992), suggesting an involvement of EGF in renal tubular regulation. A role for EGF as a growth promoting agent in kidney was also suggested by an increased EGF content of hypertrophy kidney after unilateral nephrectomy (Miller et al., 1992).

### 1.4.3 Basic Fibroblast growth factor (bFGF)

Basic FGF is a single chain polypeptide, composed of 146 amino acids (Baird and Böhlen, 1990). The bFGF gene encodes 3 exons separated by two introns. In various cultured cells and tissues, the bFGF gene encodes two polyadenylated RNAs of approximately 3.7 and 7 kb. All cell types which respond to bFGF bear specific FGF cell surface tyrosine kinase receptors.

Basic FGF has been purified from many meso-, ecto- and neuroectodermal-derived tissues, which have been shown to be FGF sensitive either *in vitro* or *in vivo* (Gospodarowicz et al., 1987). *In vitro*, bFGF is a potent mitogen for meso-, and neuroectodermal derived cells such as fibroblasts, skeletal myoblasts and vascular endothelial cells. Addition of bFGF to rat cerebral cortical neurons markedly enhanced their survival (Morrison et al., 1986) and promoted the formation of new nerve fibre tracts (Thompson et al., 1989). In early embryos, bFGF can act as a primordial differentiation factor which induces the ectoderm to become mesoderm. Infusion of anti-bFGF has significantly retarded rat embryo growth and differentiation of all tissues of endodermal origin and decreased their protein and DNA content (Liu and Nicoll, 1988). The same authors found a pronounced increase in embryonic growth, protein and DNA content following infusion of bFGF. These results suggest that bFGF may be important for growth and differentiation of endodermal and some mesodermal tissues of mammalian embryos (Liu and Nicoll, 1988). Receptors for bFGF have been detected in fetal bovine ovaries, suggesting that bFGF is a potent mitogen

involved in regulation of granulosa cell function (Wandji et al., 1992). Basic FGF and its receptor have been detected in different adult tissues including pancreas, liver and kidney (Knee et al., 1994; Friess et al., 1992). It was shown that bFGF stimulated ODC activity which is an early event in cell proliferation and increased ODC mRNA levels in a pancreatic tumoral cell line (AR4-2J) (Sarfati et al., 1992). Hoshi and Logsdon (1993) observed that bFGF stimulated DNA synthesis in a dose-dependent fashion in pancreatic acinar cells, the maximum effect being 6-7 fold over control. Basic FGF induced a marked increase in the levels of inositol 1,4,5- trisphosphate and a rapid rise in cytosolic free calcium $[Ca^{2+}]i$ levels in rat pancreas acini (Chandrasekar and Korc, 1991), indicating that bFGF may play an important role in regulation of exocrine pancreatic function. It was found that isolated mouse hepatic sinusoidal endothelial cell synthesized bFGF (Rosenbaum et al., 1989). As a potent mitogenic regulator, bFGF stimulated the proliferation of all cell types involved in the wound healing process both *in vitro* and *in vivo* (Buntrock et al., 1984; Gibran et al., 1994). Basic FGF uptake by the liver was increased after partial hepatectomy (Baruch et al., 1995), suggesting a role for bFGF in liver regeneration. Basic FGF is a known mitogen for skeletal muscle precursor cells and many other mesodermal cell types (Gospodarowicz et al., 1987) by the observation that bFGF present in muscle recovering from a crush injury revealed that bFGF plays a role in skeletal muscle regeneration (Anderson et al., 1995).

### 1.4.4 Nutritional regulation of IGFs, EGF and bFGF

Growth hormone and nutrition are two important regulators of IGF-I expression. This regulation of IGF-I may be a key control point for nutritional regulation of growth (Bass et al., 1991; Pell et al., 1993). Gene expression of IGF-I exhibits a profound sensitivity to nutritional alteration in young growing rats (Straus and Takemoto, 1990). Fasting results in a decrease in IGF-I and growth hormone receptor (GHR) mRNA levels and IGF-I gene transcription in rat liver (Straus and Takemoto, 1990). A decrease in circulating levels of IGF-I and abundance of hepatic and muscle IGF-I mRNA have been observed during periods of protein deprivation (VandeHaar et al., 1991). A RNase protection assay revealed that fasting reduced nuclear transcripts of IGF-I and another nutritionally sensitive gene, the gene encoding transthyretin (TTR) (Hayden et al., 1994). The decrease in IGF-I mRNA was matched by a similar decrease in IGF-I nuclear transcripts (Hayden et al., 1994), suggesting that fasting controls IGF-I gene expression primarily at the transcriptional level. In protein restricted rats, IGFBP-1 mRNA levels rose rapidly in the liver and kidney whereas hepatic IGF-I and IGFBP-3 mRNA decreased, thus paralleling the serum IGFBP-3 concentration (Lemozy et al., 1994). In contrast, an absence of change in IGFBP-3 mRNA and a slight increase in IGFBP-2 were observed in the kidney (Lemozy et al., 1994), suggesting that there is an organ specificity in the nutritional regulation of IGFBPs. Data about the effect of weaning on serum IGF are limited. White et al. (1991) reported that in the pig, serum IGF-I and IGFBP-3 levels decreased 36 h after weaning. Recently, Cymbaluk and Laarveld

(1996) found that in foals, weaning whether at 13 or 16 weeks of age, reduced growth rates and serum IGF-I concentrations. Serum IGF-I values recovered to preweaning values within 1 to 3 weeks postweaning concurrent to an improved weight gain.

Very little information is available about nutrition status effect on EGF and bFGF concentrations in tissue and serum during development. It was reported that nutrient intake may modulate EGF receptor number *in vivo* (Freidenberg et al., 1986). In fact, fasted rats had 40-50% less receptor binding affinity. Fasting also caused a significant decrease in gastrointestinal tissues of EGF levels in suckling rats but resulted in minimal changes in adults, suggesting that the contents of EGF in gastrointestinal tract are dependent on both age and dietary status (Schaudies et al., 1989). Levels of EGF decreased in gastrointestinal mucosa of rats fed with higher proportion of fibre (Schaudies et al., 1989), indicating that dietary fibre modulates the content of EGF. EGF receptors were widely distributed in the intestinal mucosa and submucosa of pigs. Levels of receptor tended to be higher in weaned than in newborn pigs (Kelly et al., 1992), suggesting a functional role for EGF during postnatal development.

One study showed that bFGF mRNA level was altered by diabetes in a tissue-specific fashion (Karpen et al., 1992). They found that in skeletal muscle, the bFGF mRNA level decreased, while it increased in the heart, lung and brain. As far as we know, there is no information about effect of weaning on IGFs, EGF and bFGF mRNA levels in various mammalian tissues.

## 1.5 HYPOTHESIS AND OBJECTIVES OF THE PROJECT

The above observations support the view that growth factors are regulators of fetal and postnatal growth and development. In the rat, liver is believed to be the major producer of circulating IGF-I (Daughaday and Rotwein, 1989) as well as an important endocrine source of IGFBPs (Ooi et al., 1990; Orlowski et al., 1990). The skeletal muscle represents the bulk of the body weight, which is a major target tissue for IGFs. Receptors for IGFs have indeed been found in muscle and cultured skeletal muscle cells (Beguinot et al., 1985). In the human, the kidney is the main source of urinary EGF (Gregory, 1975) and relatively high EGF concentration has been detected in the pig kidney (Vaughan et al., 1992). Pig is used in the present study because of its anatomical and physiological similarities with the human. In addition, pig pancreas is big enough to do all the planned measurements (radioimmunoassay, Western blot, Northern blot, RNA, DNA and protein concentration) simultaneously on the same animal. Although accumulating information suggests that IGFs, EGF and bFGF are involved in animal growth and development, about ontogenic IGFs mRNA levels in pig tissues studies are very limited. No information is available on the ontogeny of EGF and bFGF mRNA levels and tissue IGFs and IGFBPs concentrations in various tissues of mammals. The relationship between mRNAs and tissue concentrations of IGFs and IGFBPs has not been reported yet.

Therefore, the objectives of the present study in the pig are:

(1) to examine the ontogeny of IGFs, IGFBPs, IGFRs, EGF, EGFR and bFGF mRNA

levels as well as tissue concentrations of IGFs and IGFBPs in the pancreas, liver, kidney and skeletal muscle

(2) to examine the relationship between mRNA levels and tissue concentrations of these growth factors and organ growth during development

(3) to determine the effect of weaning on levels of IGFs, IGFRs, IGFBPs, EGF, EGFR and bFGF mRNAs in pancreas, liver, stomach, duodenum and skeletal muscle

# CHAPTER II MATERIALS, METHODS AND RESULTS

2.1 M. Peng, T. Abribat, E. Calvo, D. DeBel, M.-F. Palin, G. Bernatchez, J. Morisset and G. Pelletier. ONTOGENY OF IGFs, IGFBPs AND IGFRs mRNA LEVELS IN PORCINE PANCREAS. J. Anim. Sci. (Submitted).

## 2.1.1 Contribution to manuscript 2.1

My contribution to this manuscript was to (1) plan and write the project outline, (2) prepare and organize the sample collection, (3) extract total RNA and carry out Northern analysis, (4) provide data to the statistician, (5) prepare the figures and write the manuscript. Dr. T. Abribat's laboratory determined IGFs and IGFBPs concentrations by RIA and Western blot analysis respectively. We have also got the technical assistance of Drs. E. Calvo for RNA isolation, M-F. Palin for probe preparation. In addition, Mr. G. Bernatchez and the team of M. Morisset have contributed to samples collection. The project was directed by Drs. G. Pelletier and D. LeBel. Help in the manuscript preparation was provided by Drs. G. Pelletier, D.LeBel and J. Morisset.

2.1.2 Manuscript 2.1

Running head: Developmental IGFs mRNA in pig pancreas

# Ontogeny of IGFs, IGFBPs, IGFRs and GHR mRNA levels in porcine pancreas

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## Abstract

The ontogeny of mRNA levels of insulin-like growth factor-I and -II (IGF-I and -II, or IGFs), IGF type 1 (IGFI-R) and type II receptors (IGFII-R), IGF binding protein-1 and -3 (IGFBP-1 and -3 or IGFBPs), growth hormone receptor (GHR) and tissue concentrations of IGFs and IGFBPs were examined in the pancreas of pigs. Tissues were collected from fetuses at 90 and 110 d of gestation and pigs at 1, 21, 90 and 180 d of age. Northern blots were performed using total RNA hybridized with $^{32}P$ labelled cDNA probes (human IGF-I and human IGFI-R) and cRNA probes (rat IGF-II, human IGFII-R, human IGFBP-1, pig IGFBP-3 and pig GHR). There were two accelerated growth periods of pancreas: the first one at 90 d of fetal life, which is characterized by cell hyperplasia (high ratio of DNA to body weight) and the second one at postnatal 90 d which is attributed to cell hypertrophy (high ratios of pancreatic weight, RNA and protein to DNA). The level of IGF-II mRNA and its tissue concentration were predominant during fetal life and low thereafter. The IGF-I mRNA level was high during fetal and early postnatal life and decreased thereafter. Messenger RNA levels of IGFI-R, IGFBP-3 and GHR and concentrations of IGFBP-1 and -2 were abundant during fetal and early postnatal life. In conclusion, IGFs may be involved in various physiological periods of pancreatic development in pig.



Key Words: Pigs, Pancreas, Age, mRNA, IGF

## Introduction

It is well accepted that growth and secretion of the exocrine pancreas are dietarily and hormonally regulated. Insulin-like growth factor -I and -II (IGF-I and -II or IGFs) are ubiquitous mitogenic peptides that are present in serum and various tissues and stimulate the proliferation and differentiation of a variety of somatic cell types (Froesch et al., 1985). So far, the information on IGFs in regulating pancreatic development have been derived largely from rodents. It was reported that IGFs stimulate DNA synthesis in isolated fetal rat islets of Langerhans (Hogg et al., 1993). However, no data is available on tissue concentration and mRNA content of IGFs in pancreas of large mammals during development.

The physiological effects of IGFs are mediated predominantly by the type I IGF receptor (IGFI-R) which binds IGF-I with greater affinity than IGF-II (Nissley and Lopaczynski, 1991). IGF-II also binds to the IGF-II/Mannose-6-phosphate receptor (Type II IGF receptor or IGFII-R). Both IGF receptor subtypes are widely distributed into the different organisms of the body (Nissley and Lopaczynski, 1991; Werner et al., 1991). IGFI-R was also found on rat pancreatic A and B cells (Van Schravendijk et al., 1987).

Messenger RNAs for IGF-I and -II were found in various tissues of rat (Murphy et al., 1987) and pig (Lee etal., 1993; Peng et al., 1996a). Tissue mRNA levels of IGF-I and -II were developmentally regulated in both rat and pig.

In serum and other extracellular fluids, IGFs are associated with specific IGF binding proteins (IGFBPs) which are thought to prolong their half-life, serve as their carrier in the

circulation, provide a storage pool for IGFs or act as regulator of their biological actions by regulating their availability to cell surface receptors (Clemmons, 1991). Various forms of IGFBPs have been identified from pig serum (Lee et al., 1991a). In human, IGFBP-1, -2 and -3 were found in various fetal tissues, including the pancreatic endocrine tissue (Hill and Clemmons, 1992). Exogenous IGFBP-1 and -2 can potentiate the ability of IGF-1 or IGF-II to stimulate DNA synthesis by isolated fetal rat islets of Langerhans (Hogg et al., 1993). In rat, pancreatic IGFBP-1 and -2 mRNAs increased from late fetal to early postnatal life, whereas mRNAs for IGFBP-3 and -4 were predominant after birth (Hogg et al., 1994).

Growth hormone (GH) plays an important role in postnatal development. The GH acts via its membrane-bound receptor and mRNA of GH receptor (GHR) has been found in fetal rat tissues (Walker et al., 1992) and in liver and muscle of pig (Brameld et al., 1996).

The objectives of the present study were (1) to characterize the developmental pattern of pancreatic growth, (2) to determine mRNA levels of IGFs, IGFBPs, IGFRs and GHR and tissue concentrations of IGFs and IGFBPs in the pancreas during fetal and postnatal development and (3) to investigate potential relationship that might exist between pancreas growth, mRNA levels and tissue concentrations of IGFs and IGFBPs in pig.

## Materials and Methods

*Tissue Preparation.* Yorkshire purebred fetuses from 90 (F90) and 110 (F110) d of gestation and pigs of postnatal 1 (P1), 21 (P21), 90 (P90) and 180 (P180) d of age were used. Ages of the fetuses were determined according to the sows mating date. For each age, two males and two females were obtained from the same litter and this was replicated three times. Pigs including sows, with the exception of fetuses were fasted for 16 h. Animals were slaughtered (stunning and exsanguination) according to recommended code of practice for the care and handling of farm animals, pigs (Agriculture and Agri-Food Canada, 1993). After laparotomy, the uterus was gradually opened and the fetuses were removed. The body weights (mean ± SE) at slaughter were .61±.01, 1.15±.05, 1.48±.03, 6.08±.46, 28.9±2.12 and 95.4±3.48 kg for F90, F110, P1, P21, P90 and P180 respectively. Immediately after exsanguination, the pancreas was removed by dissection and weighted. Tissue samples used for Northern blot analysis and radioimmunoassay (RIA) measurements were frozen in liquid nitrogen and stored at -80°C until analyzed. Fresh tissue samples were used for DNA, RNA, protein determination and enzyme activity assays.

*Measurement of DNA, RNA and Protein.* Tissues (150 mg) were homogenized and precipitated in .7 N perchloric acid (PCA) for DNA, RNA and protein determinations. Protein was analyzed by the method of Lowry et al. (1951) with bovine serum albumin (BSA) as a standard. DNA and RNA were extracted and measured according to the methods previously described (de Passillé et al., 1989).

*RIA.* Pancreas IGF-I and -II were quantified by RIA using methodologies previously described (Abribat et al., 1990; Lee et al., 1991b). The pancreatic tissue was homogenized in freshly prepared 3.3 M formic acid containing .5% Tween 20 (1:4 wt vol) at pH 2 and centrifuged at 40,000 × g for 15 min at 4°C to dissociate the IGF-IGFBPs complex. A 150 μL aliquot of each sample was heated at 90°C for 30 min, 350 μL acetone were then added and the mixture was thoroughly vortexed followed by centrifugation at 3,500 × g for 15 min at 4°C. The supernatant was neutralized with the RIA buffer and used directly for IGFs measurement.

Recombinant human IGF-I and II (Bachem California, Torrance, CA) were used for iodination and standards. Anti hIGF-I antibodies were kindly provided by Drs E. Underwood and J.J. Van Wyk (University of North Carolina, by the National Institute of Diabetes, Digestive and Kidney Disease through the National Hormone and Pituitary Program). Monoclonal anti-hIGF-II was purchased from Amano International Enzyme Co (Troy, VA). The RIAs were carried out under equilibrium condition using IGF-I or -II radioiodionated by conventional chloramine T method. The RIA buffer consisted of 30 mM phosphate buffer, pH 7.4, containing .02% protamine sulphate (Grade II), 10 mM EDTA, .02% sodium azide and .25% bovine serum albumin (BSA) RIA grade (all buffer components from Sigma Chemical Co, St Louis, MO). One hundred μL of diluted sample extract or standard were incubated at 4°C for 24 h with 100 μL of specific antibody (final dilution 1/20,000 and 1/50,000 for the anti IGF-I and IGF-II antisera, respectively, 100 μL $^{125}$I-IGF-I or -II tracer (10,000 cpm) and 200 μL of RIA buffer. The binding reaction was stopped by precipitation

(24 h at 4°C) with 100 $\mu$L of the second antibody (goat antirabbit IgF for IGF-I and goat antimouse IgG for IGF-II purchased from Linco Research Inc., St. Louis, Mo) and pellets were counted in a gamma counter (Beckman LS 580, Beckman Instruments Inc., Scientific Instruments Division, Irvine, CA) after centrifugation. In our assay conditions, ED50s from standard curves were 60 $\pm$ 8 pg/tube and 86 $\pm$ 10 pg/tube for IGF-I and -II, respectively (means $\pm$ SD of 10 replications). Intra and inter assay coefficients of variation were 4 and 13% and 3 and 12% for IGF-I and IGF-II, respectively.

*Western Ligand Blotting for IGFBPs.* Pancreatic acid extracts were subjected to Western ligand blot analysis as described by Hossenlopp et al. (1986). Initial experiments showed that neutralized acid extracts gave similar results to extracts performed in neutral conditions. Therefore, the 40,000 $\times$ g supernatants of the formic acid extracts (described in the previous section) were neutralized with an equal volume of 2 M Tris buffer, pH 8.5, then diluted in SDS non reducing sample buffer, and loaded onto a 12% SDS polyacrylamide gel and electrophoresed. Proteins were then transferred to nitrocellulose (BioRad, Richmond, CA) at 15 V overnight in a Transblot Cell (BioRad, Richmond, CA). The blotted membranes were washed with Tris buffer saline (TBS) (.1 M Tris, pH 7.4, .15 M NaCl, .05% sodium azide), containing sequentially 3% NP 40 for 30 min, 1% BSA for 2 h, .1% Tween 20 for 10 min and then incubated with $^{125}$I-IGF-I ($1.4\times10^5$ cpm/ml) in 30 ml TBS, .1% BSA overnight at 4°C. Membranes were washed twice for 15 min in TBS, .1% Tween 20, three times for 15 min in TBS, dried and exposed to X ray films in the presence of intensifying screen for 3 days at -80°C. For quantitative analysis, bands of the membrane corresponding

to those on autoradiograms were cut and counted in a gamma counter. For each individual IGFBP, data were expressed as cpm.

*RNA Preparation.* Total RNA from the pancreas were extracted by the method described by Chirgwin et al. (1979). Tissues were homogenized in 4 M guanidinium thiocyanate using a polytron homogenizer. Supernatants were used for precipitation of RNA in 100% ethanol and 2 M potassium acetate at -20°C. After centrifugation, the RNA pellets were dissolved in 7 M guanidine hydrochloride. Then, RNA was precipitated again and quantified by measuring its absorbance at 260 nm.

*Preparation of Probes.* A human IGF-I cDNA which corresponds to a 659 bp cDNA *Eco*R1 fragment (Jansen et al., 1983) and a human IGF-IR cDNA (Ullrich et al., 1986) corresponding to a 700 bp cDNA *Eco*R1 fragment were used. The cDNA probes were labelled with [$^{32}$P]dCTP (3000 Ci/mmol; Amersham Canada Limited, Oakville, Ont., Canada) by random priming using a commercial kit (Megaprime DNA labelling System, Amersham Canada Limited, Oakville, Ont., Canada).

A rat IGF-II, human IGFBP-1, porcine IGFBP-3 and human IGFII-R complementary RNA (cRNA) probes were transcribed from a 720 base pairs (bp) *Bam*H1 cDNA fragment (Ueno et al., 1988), a 1443 bp *Eco*R1 cDNA fragment (Julkunen et al., 1988), a 595 bp *Eco*R1-*Sma*I cDNA fragment (Shimasaki et al., 1990) and a 695 bp *Kpn*I-*Hind* III (Morgan et al., 1987) fragment, respectively. The cDNA fragments were inserted into the pBluescript KSII+ plasmid vector. The inserts were sequenced using a T7Sequencing™ Kit (Pharmacia Biotech Inc, Piscataway, NJ) in order to check the orientation of the cDNAs. The templates

were linearized with *Eco*R1 for IGF-II, *Hind*III for IGFBP-1, *Sam*1 for IGFBP-3 and *Kpn*I for IGFII-R and then used in a T7 transcription Riboprobe System (Promega, Madison, WI).

The GHR probe was prepared by RT-PCR. The GHR primers were designed according to known regions of the pig GHR cDNA sequence (Cioffi et al., 1990). The upstream primer (5'-ATCCCGCGGAACCACCTCCCAATGCAGATGTTC-3') is identical to nucleotide 545-568, while the downstream primer (5'-ATCTCTAGATGGCGCTAGACGTGATTCAACCTC-3') represents the reverse complement of nucleotides 1669-1692. An *Eco*RV site for upstream primer and a *Xba*I site for downstream primer were created by addition of nine nucleotides. This primer pair predicts a 1166 bp fragment. First-strand cDNA was synthesized from total RNA (6 $\mu$g) using a "SuperScript™ Preamplification System for First Strand cDNA Synthesis" kit (Gibco BRL, Life Technologies, Burlington, ON) and 500 ng of oligo$(dT)_{12-18}$ primer in a total reaction volume of 30 $\mu$L. An aliquot of 3 $\mu$L was subjected to PCR amplification using a PTC-100™ Programmable Thermal Controller (MJ Research Inc., Watertown, Mass.). The reaction mix contained 3 $\mu$L of reverse transcription mixture, 30 pmol of each primer, 1x PCR buffer (Pharmacia Biotech Inc., Piscataway, NJ), .35 mM dNTPs and water to 100 $\mu$L. After overlaying with 3 drops of light mineral oil (Sigma ST. Louis, MO), the reactions were heated to 94°C for 2 min prior to the addition of 4 U of the *Taq* polymerase (Pharmacia Biotech Inc., Piscataway, NJ). The amplification profile comprised 35 cycles: at 94°C for .75 min (dissociation), 55°C for 1.25 min (annealing) and 72°C for 2.5 min (extension). The final cycle included a further 5 min at 72°C for complete strand extension. A PCR amplified

cDNA fragment of the predicted length was inserted into the pBluescript KSII+ plasmid vector. The authenticity of the cDNA clone was verified by sequencing using a T7Sequencing™ kit (Pharmacia Biotech Inc., Piscataway, NJ). The template was linearized with *Eco*RV and then used in a T7 transcription Riboprobe System (Promega, Madison, WI) to synthesis the cRNA probe.

*Northern Blot Analysis.* The procedure for Northern blot was described by Lehrach et al. (1977) with some modifications. Briefly, 20 $\mu$g of total RNA was fractionated in a 1% agarose gel containing 2.2 M formaldehyde before transfer to nylon membranes (Nytran, Schleicher and Schuell, Keene, NH). For RNA probes, membranes were prehybridized for 4 h in a solution of 50% formamide, 5 × SSC, 50 mM Hepes pH 6.8, 2 mM EDTA pH 8.0, 5 × Denhardt's, 1% SDS and 200 $\mu$g/ml of salmon sperm DNA at 65°C. They were then hybridized in the same solution overnight at 65°C with cRNA riboprobes (rat IGF-II, human IGFII-R, human IGFBP-1, porcine IGFBP-3 and pig GHR) which were labelled with [$^{32}$P]-labelled dUTP.

For DNA probes, membranes were prehybridized for 15 min in a solution (Rapid-hyb buffer, Amersham Canada Limited, Oakville, Ont., Canada) at 65°C, and then hybridized in the same solution for 2 h at 65°C with cDNA probes labelled with [$^{32}$P]-labelled dCTP (human IGF-I and human IGF-I receptor). Membranes were then washed with .1 × SSC/ .1% SDS twice, 30 min at 85°C for RNA probes and at 60°C for cDNA probes and exposed to Kodak films (Eastman Kodak Company, Rochester, NY) with an intensifying screen at -80°C. The blots were reprobed with 18S ribosomal $^{32}$P-labelled cDNA probe as a control to

quantitate RNA loading and transfer. The size of RNA transcript was evaluated according to the position of 18S and 28S rRNAs or RNA ladder.

Autoradiograms were scanned using a densitometer (BIO-RAD Imaging Densitometer Model GS-670, Biorad Laboratories Led., Mississauga, Ont., Canada). The relative density of the transcript is expressed as arbitrary optical units. To correct for the possible difference in loading of total RNA in Northern blot, a ratio of the relative density of each specific transcript of growth factors with the relative density of the 18S ribosomal RNA band was calculated before statistical analysis was performed.

*Statistical Analysis*. We ran an analysis of variance for the following parameters: body weight, organ weight, tissue content of RNA, DNA and protein. We used the age of pig as main effect, sow within each age as first error term, sex of piglets as a split effect and age × sex interaction. The different ages were compared using a *priori test* comparisons. The following comparison were used: (1) fetuses (F90 + F110) vs suckling piglets (P1 + P21), (2) suckling vs postweaning pigs (P90 + P180), (3) within fetal ages: F90 vs F110, (4) within suckling ages: P1 vs P21, (5) within postweaning ages: P90 vs P180. For the Northern blot analysis, the same model as the previous one was used, except that the membrane effect was tested instead of the sow effect. All variables were submitted to an analysis of variance using the general linear model procedure of Statistical Analysis System software (SAS, 1985). Data were considered significantly different if probability value $(P) \leq .05$.

## Results

*Development of the Pancreas*

Two fast growing periods of the pancreas in terms of organ weight to body weight were observed at fetal F90 and P90 respectively (Figure 1a). The highest ratio of total pancreatic DNA to body weight was observed at F90 and decreased ($P < .001$) to a lower level thereafter (Figure 1b). The ratios of pancreatic weight to DNA, RNA to DNA and protein to DNA were the lowest ($P < .001$) during the fetal life and increased thereafter (Figure 1c, d and e).

*Pancreatic IGFs, IGFBPs, IGFRs and GHR mRNA Levels*

One major IGF-I mRNA transcript of approximately 7.8 kb was detected in the pancreas (Figure 2, top panel). High levels of IGF-I mRNA were observed during fetal and suckling periods (P1 and P21) and decreased ($P = .001$) after weaning (P90 and P180) (Figure 2, bottom panel). Messenger RNA for IGFI-R was present as a single 11 kb transcript (Figure 3, top panel) while IGFII-R mRNA was undetectable in the pancreas (data not shown). Pancreatic mRNA of IGFI-R was relatively abundant during the fetal and neonatal life and was low from P21 to P180 ($P = .05$, Figure 3, bottom panel). A single IGF-II mRNA transcript of approximately 2.2 kb was found in the pancreas (Figure 4, top panel). The highest level of IGF-II mRNA was observed during the fetal life and decreased ($P < .05$) at birth (Figure 4, bottom panel). A 1.8 kb IGFBP-1 mRNA transcript was found abundant

in the fetal liver while it is low in the pancreas (Figure 5). A single 2.8 kb mRNA transcript for IGFBP-3 was detected in the pancreas (Figure 6, top panel). A high level of IGFBP-3 mRNA occurred from fetal to early postnatal life and declined ($P = .003$) thereafter (Figure 6, bottom panel). One major transcript of 4.6 kb for GHR was found in the pancreas (Figure 7, top panel) at a relatively high level in fetuses and newborns and was low ($P < .05$) from P21 to P180 (Figure 7, bottom panel).

*Pancreatic IGFs and IGFBPs Concentrations*

The pancreatic concentration of IGF-I was low in fetuses and newborns then peaked in piglets of 21 days of age (17 fold higher in P21 compared to F90, $P < .001$) followed by a relatively low level thereafter (Figure 8). On the other hand, IGF-II concentration was high during fetal life and decreased ($P < .001$) with advancing age (Figure 8).

Western blotting of IGFBPs in pancreas homogenates showed two major bands (Figure 9, top panel). One band was present at 32.5 kDa corresponding to IGFBP-2 and another at 27.5 kDa corresponding to IGFBP-1 which was the predominant one in the pig pancreas. Contents of IGFBP-1 and 2 shared a similar pattern with the highest levels ($P < .001$) present in fetuses then followed by a marked decrease (IGFBP-1, $P < .001$; IGFBP-2, $P = .001$) during the postnatal period (Figure 9, bottom panel). The concentrations of IGFBP-3 and 4 were barely detected (data not shown).

## Discussion

This is the first report on the development of IGFs, IGFRs, IGFBP-1 and -3 mRNA levels and tissue concentrations of IGFs and IGFBPs in the pancreas of pig. A fast fetal pancreatic growth at F90 is characterized by cell hyperplasia (high DNA / body weight) and a fast postnatal growth at P90 can be attributed to cell hypertrophy (high RNA / DNA and protein / DNA). The rapid growth of the pancreas during fetal life was accompanied by high levels of IGF-II and IGFI-R mRNAs and high tissue IGF-II concentration in the pancreas. It was reported that mouse embryos carrying null mutations of the genes encoding IGF-I, -II and IGFI-R lead to growth deficiency (Baker et al., 1993). Furthermore, using *in-situ* hybridization, IGF-II mRNA was found more abundant than that of IGF-I in human fetal exocrine and endocrine pancreas (Miettinen et al., 1993). However, in the current study, IGFII-R mRNA was undetected. Baker et al. (1993) reported that in 11 and 12.5 day-old mouse embryo, IGFI-R mediates only mitogenic signalling of IGF-II. After 13.5 days of age, IGFI-R interacts with both IGF-I and -II while IGF-II recognizes an additional unknown receptor which is different from IGFII-R. This suggests that IGF-II regulates pancreatic growth mainly through IGFI-R.

The pancreas IGF-I concentration was the highest at 21 days of age which is preceded by a fast growth period of pancreas at P90. In the present study, we did not evaluate changes of pancreas weight between 21 to 90 days of age, although another fast growth period of this organ occurred at 90 days of age. However, it is possible that pancreatic weight increased

before 90 days of age. This assumption is supported by the fact that pancreatic weight (Peng et al., 1996b) and pancreatic enzyme activities increased during the weaning period (Kelly et al., 1991; Lainé et al., 1996). A role of IGF-I on pancreatic growth was suggested since IGF-I is present in pancreatic A cells while IGF-II was observed exclusively in B-cells in man, and the pancreas of dog and rat (Maake and Reinecke, 1993). It was also observed that IGF-I mRNA increased fourfold above control value 3 days after 90% of rat pancreatectomy (Smith et al., 1991) and high affinity receptors for IGF-I were present on rat pancreatic A and B cells (Van Schravendijk et al., 1987). We have observed that IGF-I and IGFI-R mRNAs were more abundant in the pancreas during the fetal life than during the postnatal life. However, IGF-I mRNA is much less abundant in pancreas compared to the other studied tissues (unpublished observations). This is consistent with a study on human (Bergmann et al., 1995), where IGF-I mRNA was low in normal pancreas. Therefore, during postnatal life, pancreatic growth of the pig might also be regulated by other growth factors. The cholecystokinin (CCK) is a gastrointestinal hormone that plays an important role in regulation of pancreatic function and stimulate pancreatic growth (Williams and Blevins, 1993). However, a previous study indicated that pig pancreatic acinar cells express few $CCK_A$ receptors and possess a large majority of the $CCK_B$ receptors responsible for their relatively low sensitivity to CCK (Morisset et al., 1996). On the other hand, Vilá et al. (1995) using [$^3$H]-thymine uptake assay, found that hepatocyte growth factor (HGF) was the most potent mitogen for normal human pancreas cultures compared to IGF-I and EGF. The HGF receptor has been identified as *c-met* which was located to the apical membrane of

ductal cells in normal pancreas tissue (Vilá et al., 1995), suggesting that HGF and *c-met* may be involved in pancreatic growth. In the present study, IGF-I mRNA was abundant in fetal and early postnatal life and decreased with advancing age which is in agreement with our recent observations in rats (Calvo et al., 1996). However, these observations are not in agreement with the study reported earlier by Hogg et al. (1994), who showed that pancreatic IGF-I mRNA was predominant during late postnatal development in rats. The reason for such a discrepancy is unknown, beside the fact that different breeds of rat were used in these studies.

The levels of IGF-I and IGFBP-3 mRNA were high during the fetal and neonatal life while IGF-I concentration was low during the same period and IGFBP-3 was barely detectable over the whole developmental period examined. These results suggest that tissue content of IGF-I and IGFBP-3 may be regulated by posttranscriptional and(or) translational rate of protein synthesis. The levels of IGFs, IGFI-R, IGFBP-3 and GHR mRNAs presented a similar pattern which was high during the fetal and early postnatal life and declined thereafter, suggesting that accumulation of these mRNAs is coordinated in the pancreas. The IGFBP-3 is believed to be the major carrier of IGFs in the serum of adult pig (Lee et al., 1991a). In cell culture, intact IGFBP-3 has been shown to directly alter the interaction between IGF-I and its receptor (McCusker et al., 1991). Furthermore, it was reported that during rat pregnancy, IGFBP-3 decreased in serum, fetus and reproductive tissues. The decreased in IGFBP-3 was temporally related to the appearance of IGFBP-3 protease activity (Davenport et al., 1992). The undetected IGFBP-3 level in fetal pancreas may also be due

to protein degradation and(or) proteolysis of IGFBP-3 which likely makes more IGF-I and -II available for binding to cell surface receptors.

In the present study, pancreatic GHR mRNA was abundant in fetuses and newborns and decreased thereafter. Messenger RNA for GH receptor/binding protein (GHR/BP) (Edmondson et al., 1995) and GH receptors (Walker et al., 1992) have been identified in rat fetal tissues including the pancreas. In the fetal rat pancreas, GHR/BP mRNA was expressed mainly in the epithelium cells of the pancreatic acini which is adjacent to the connective tissue expressing high level of IGF-I mRNA, thus suggesting a role for GH acting both directly and indirectly via IGF-I (Edmondson et al., 1995). In addition, it was reported that GH stimulates DNA replication of fetal rat islet cell directly by inducing the local production of IGF-I (Swenne et al., 1987), an indication that GHRs detected in *vivo* are functional. Together these findings and data of the present study suggest that IGF-I may act as a mediator of GH involved in fetal pancreatic growth.

We observed that pancreatic IGFBP-1 and -2 contents were high during fetal and early postnatal life, thus parallelling the high levels of pig serum IGFBP-1 and -2 (Lee et al., 1991a). However, IGFBP-1 mRNA was very low in the pancreas during the whole developmental period, suggesting that pancreatic accumulation of IGFBP-1 is regulated at the posttranscriptional level. It is also possible that high pancreatic IGFBP-1 and -2 concentrations come from serum, since these proteins can cross the capillary boundaries (Bar et al., 1990). Exogenous IGFBP-1 and -2 could both potentiate the ability of IGF-I or -II to stimulate DNA synthesis by isolated fetal rat islets of Langerhans, and that IGFBPs were

themselves released by islets *in vitro* (Hogg et al., 1993). These results suggest that IGFs and IGFBPs may interact to promote cell hyperplasia in fetuses during late gestation.

In summary, pancreatic IGFs and IGFBPs concentrations and IGFs, IGFBPs and IGFI-R mRNAs levels are developmentally regulated. Pancreatic mRNA and tissue concentration of IGF-II were abundant in fetuses and neonatal piglets. The IGF-I mRNA level was high during the fetal and early postnatal life. A peak of IGF-I concentration was found at 21 days of age corresponding to high IGF-I mRNA level and an increase of digestive enzyme activities, suggesting that IGF-I may be involved in pancreatic development.

## Implication

Our study shows for the first time a relationship between mRNA levels and concentrations of IGFs and growth of pancreas in pig. High concentrations of IGF-II and IGF-I occurred in fetal and early postnatal period respectively, which are related to a fast growth period of the pancreas and an increased enzyme activities. In addition, IGFs, IGFI-R, IGFBP-3 and GHR mRNA levels were abundant during fetal and early postnatal periods, implicating that these hormonal factors might be involved in early pancreatic growth and development in pig. Any treatment that would increase IGF production may accelerate pancreas development and pig performance around the weaning period.

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Figure 1. Effect of age on: a) pig pancreatic weight, b) total pig pancreatic DNA to body weight ratio, c) organ weight, d) total RNA, and e) protein to DNA ratios (F: fetus, P: postnatal). Values are means ± SE of 6 to 12 observations at each age (at least one male and one female per litter from three different litters were used).

Figure 2. Effect of age on IGF-I mRNA level in pig pancreas (F: fetus, P: postnatal). TOP PANEL: Representative Northern blot of IGF-I mRNA in pig pancreas during development. Total RNA (20 $\mu$g) was applied to a 1% agarose-formaldehyde gel followed by blot hybridization at 65°C using a human IGF-I cDNA probe and washed twice at 60°C with .1 × SSC/ .1% SDS for 30 min. Autoradiography proceeded for two weeks at -80°C. The size of the IGF-I mRNA transcript was estimated according to the position of 18S and 28S. Blots were reprobed with an 18S ribosomal $^{32}$P-labelled probe as a control to quantitate RNA loading and transfer. BOTTOM PANEL: IGF-I mRNA level was evaluated by densitometric scanning of the autoradiography and was expressed in arbitrary optical unit as a ratio of the 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Data at each age represent means ± SE obtained from four observations (one male and one female per litter from two different litters).

Figure 3. Effect of age on pig pancreatic IGFI-R mRNA level (F: fetus, P: postnatal). TOP PANEL: Representative Northern blot of IGFI-R mRNA in pig pancreas during development. Total RNA (20 $\mu$g) was applied to a 1% agarose-formaldehyde gel followed

by blot hybridization at 65°C using a human IGFI-R cDNA probe and washed twice at 60°C with .1 × SSC/ .1% SDS for 30 min. Autoradiography proceeded for two weeks at -80°C. The size of the IGFI-R mRNA transcript was estimated according to the position of 18S and 28S. The blots were reprobed with a 18S ribosomal $^{32}$P-labelled probe as a control to quantitate RNA loading and transfer. BOTTOM PANEL: IGFI-R mRNA level was evaluated by densitometric scanning of the autoradiography and was expressed in arbitrary optical unit as a ratio of the 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Data at each age represent means ± SE obtained from four observations (one male and one female per litter from two different litters).

Figure 4. Effect of age on IGF-II mRNA level in pig pancreas (F: fetus, P: postnatal). TOP PANEL: Representative Northern blot of IGF-II mRNA in pig pancreas during development. Total RNA (20 μg) was applied to a 1% agarose-formaldehyde gel followed by blot hybridization at 65°C using a rat IGF-II cRNA probe and washed twice at 85°C with 0.1 × SSC/ .1% SDS for 30 min. Autoradiography proceeded for one day at -80°C. The size of the IGF-II mRNA transcript was estimated according to the position of 18S and 28S. The blots were reprobed with a 18S ribosomal $^{32}$P-labelled probe as a control to quantitate RNA loading and transfer. BOTTOM PANEL: Relative tissue level of IGF-II mRNA was evaluated by densitometric scanning of the autoradiography and was expressed in arbitrary optical unit as a ratio of the 18S ribosomal RNA signal. One unit is attributed to the

maximum ratio and the other values are reported relative to this maximum ratio. Data at each age represent means ± SE values obtained from four observations (one male and one female per litter from two different litters).

Figure 5. Representative Northern blot of IGFBP-1 mRNA in various pig tissues at fetal 90 days of age (P: pancreas, L: liver, K: kidney, M: muscle). Total RNA was applied to 1% agarose-formaldehyde gel followed by blot hybridization using a human IGFBP-1 RNA probe (at 65°C). The filter was washed twice at 85°C with .1× SSC/.1% SDS for 30 min. Autoradiography proceeded approximately for two weeks at -80°C. The size of the transcript was estimated according to a RNA ladder. The blot was reprobed with a 18S ribosomal $^{32}$P-labelled probe as a control to quantitate RNA loading and transfer.

Figure 6. Effect of age on pig pancreatic IGFBP-3 mRNA level (F: fetus, P: postnatal). TOP PANEL: Representative Northern blot of IGFBP-3 mRNA in pig pancreas during development. Total RNA (20 μg) was applied to a 1% agarose-formaldehyde gel followed by blot hybridization at 65°C using a porcine IGFBP-3 cRNA probe and washed twice at 85°C with .1 × SSC/ .1% SDS for 30 min. Autoradiography proceeded for one week at -80°C. The size of the IGFBP-3 mRNA transcript was estimated according to the position of 18S and 28S. The blots were reprobed with a 18S ribosomal $^{32}$P-labelled probe as a control to quantitate RNA loading and transfer. BOTTOM PANEL: IGFBP-3 mRNA level was evaluated by densitometric scanning of the autoradiography and was expressed in arbitrary

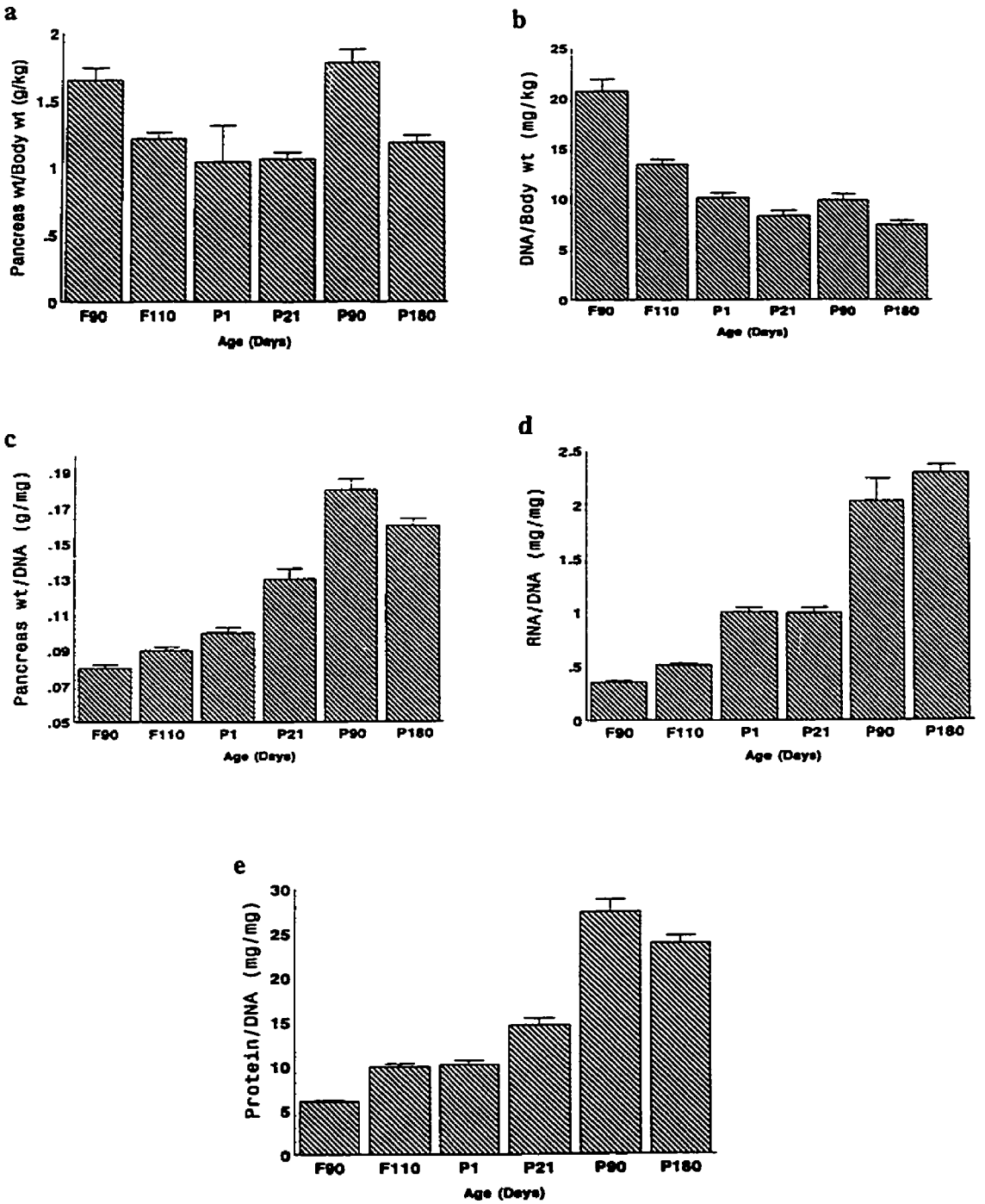
optical unit as a ratio of the 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Data at each age represent means ± SE obtained from four observations (one male and one female per litter from two different litters).

Figure 7. Effect of age on pig pancreatic GHR mRNA level (F: fetus, P: postnatal). TOP PANEL: Representative Northern blot of GHR mRNA in pig pancreas during development. Total RNA (20 $\mu$g) was applied to a 1% agarose-formaldehyde gel followed by blot hybridization at 65°C using a pig GHR cRNA probe and washed twice at 85°C with .1 × SSC/ .1% SDS for 30 min. Autoradiography proceeded for two weeks at -80°C. The size of the GHR mRNA transcript was estimated according to a RNA ladder. The blots were reprobed with a 18S ribosomal $^{32}$P-labelled probe as a control to quantitate RNA loading and transfer. BOTTOM PANEL: GHR mRNA level was evaluated by densitometric scanning of the autoradiography and was expressed in arbitrary optical unit as a ratio of the 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Data at each age represent means ± SE obtained from four observations (one male and one female per litter from two different litters).

Figure 8. Effect of age on pig pancreatic IGF-I and -II concentrations (ng/g of tissue) (F: fetus, P: postnatal). Values are means of 6 to 12 observations at each age (at least one male and one female per litter from three different litters were used). IGF-I and IGF-II

concentrations were determined by RIA from pancreas homogenates prepared by homogenization of 500 mg to 1 g of tissue in a 1 g to 4 ml ratio wt/vol of 3.3 M formic acid solution to dissociate the IGF-IGFBPs complex.

Figure 9. Effect of age on IGFBPs contents in pig pancreas (F: fetus, P: postnatal). TOP PANEL: Representative Western blotting of IGFBPs in pancreas homogenates prepared as described in Figure 8. Pancreas homogenates were separated on a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis under non-reducing conditions and transferred onto nitrocellulose membrane. Blots were probed with $^{125}$I-labelled IGF-I and autoradiographed at -80°C for 72 h. Bands of the membrane corresponding to those on the autoradiograms were counted in a gamma counter and expressed in cpm. BOTTOM PANEL: Average values of IGFBPs from three Western blots are described in top panel (IGFBP-1=27.5 kDa; IGFBP-2=32.5 kDa; IGFBP-3=39.0 kDa; IGFBP-4=26.0 kDa).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1
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0
F90
F110
P1
P21
P90
P180
Age (Days)
b
DNA/Body wt (mg/kg)
25
20
15
10
5
0
F90
F110
P1
P21
P90
P180
Age (Days)
c
Pancreas wt/DNA (g/mg)
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.09
.07
.05
F90
F110
P1
P21
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P180
Age (Days)
d
RNA/DNA (mg/mg)
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2
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F90
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P21
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Age (Days)
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Protein/DNA (mg/mg)
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Age (Days)

Figure 1.

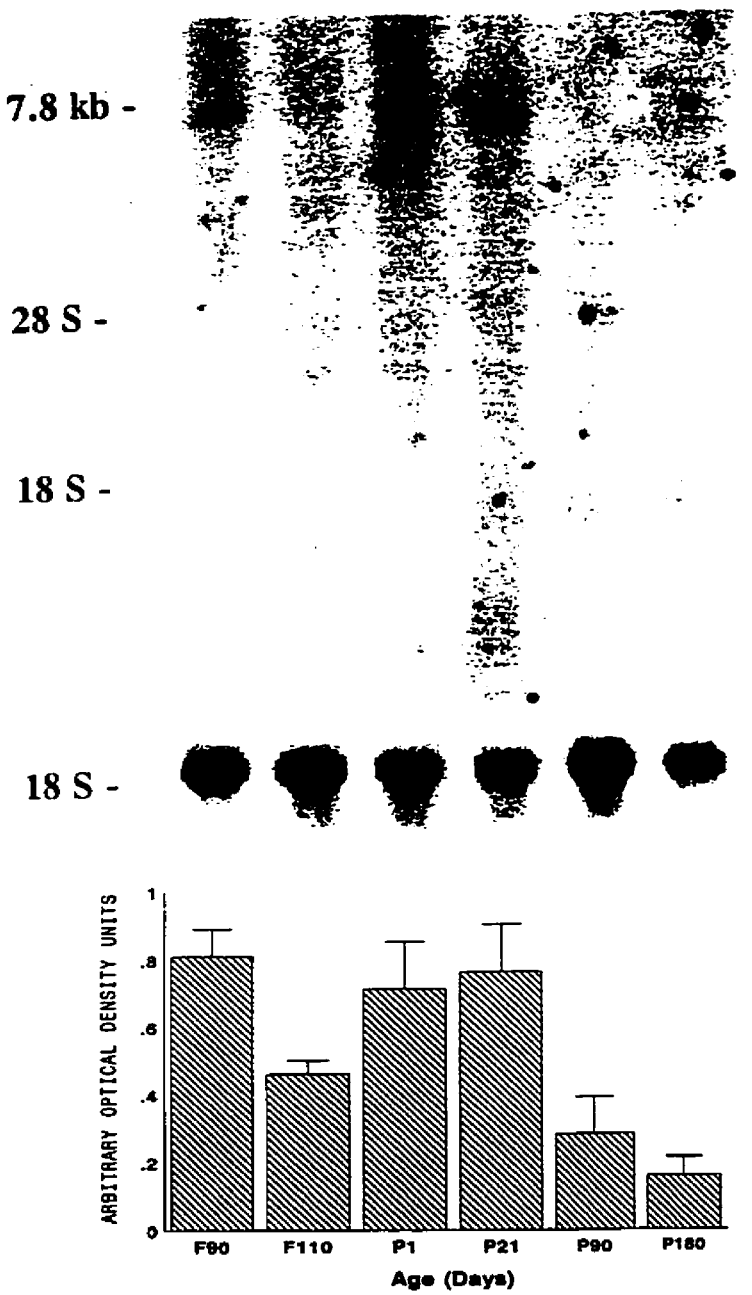
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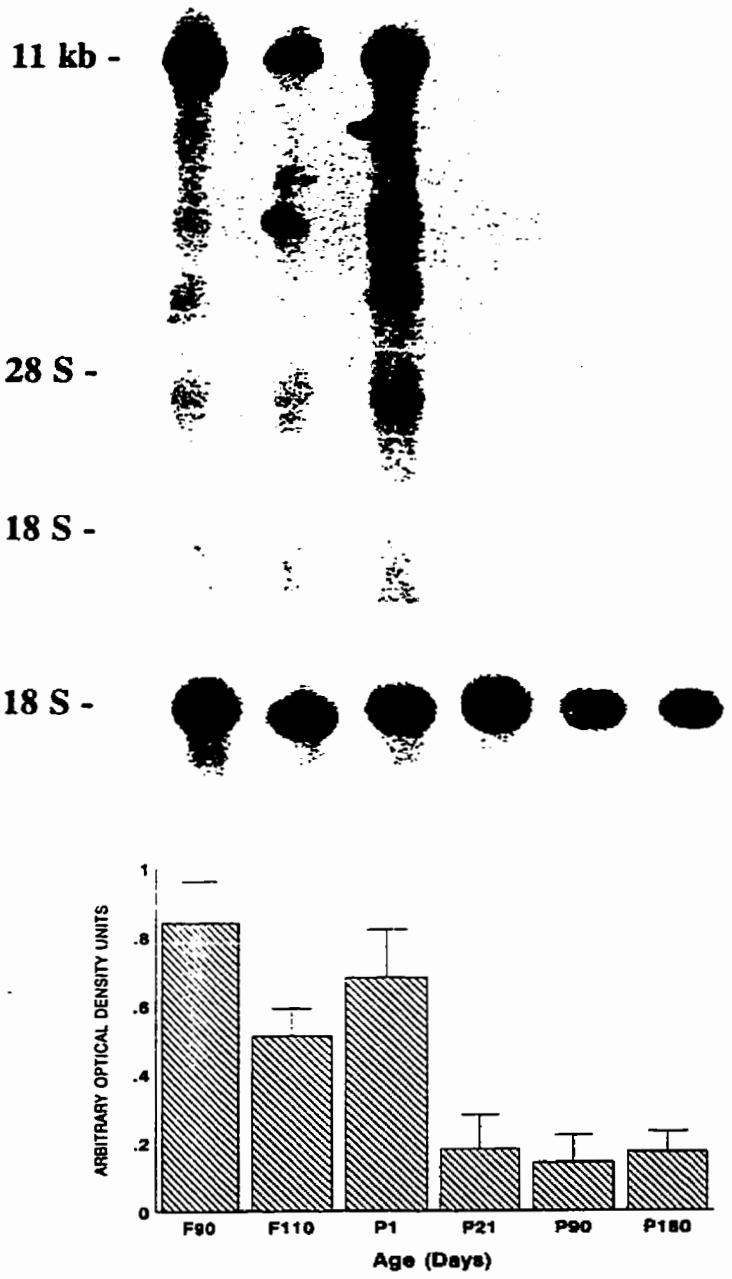
Figure 2.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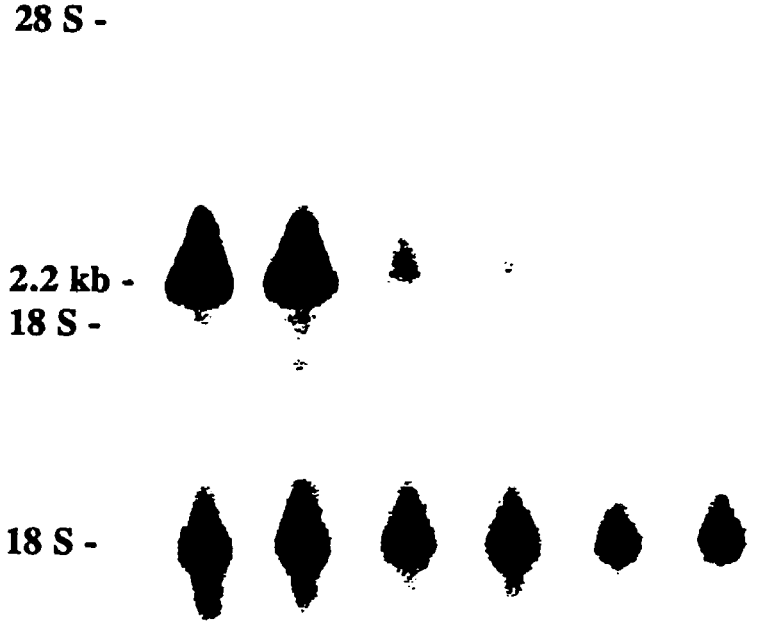
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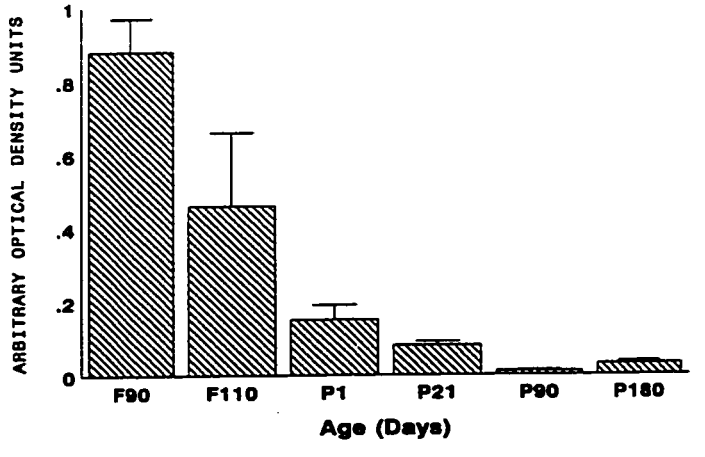
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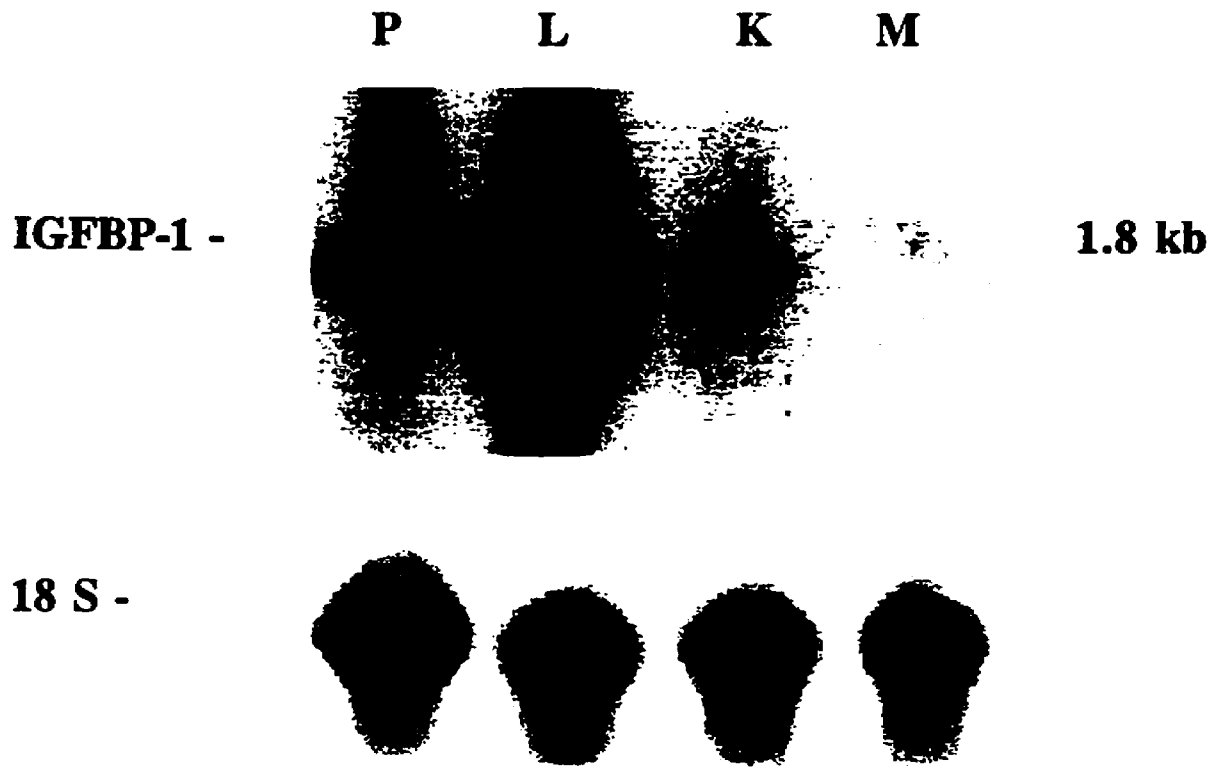
Figure 4.

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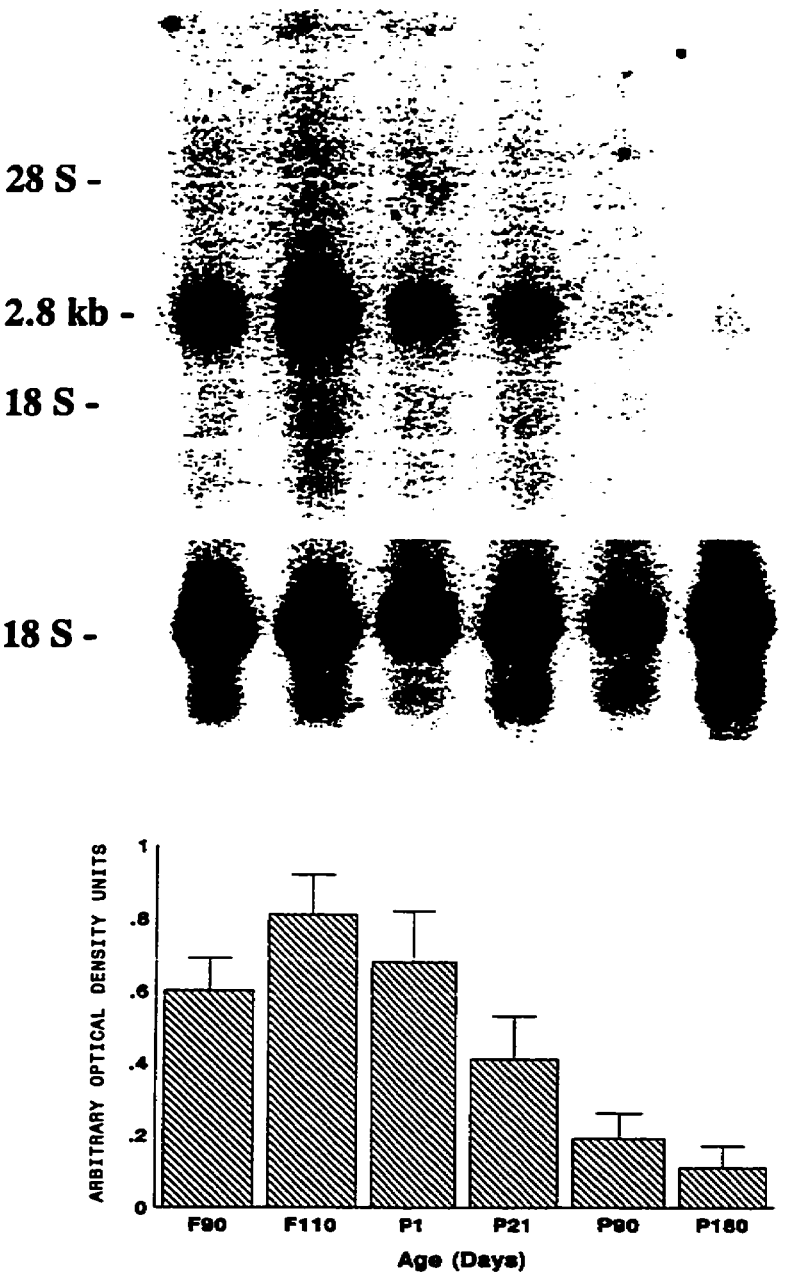
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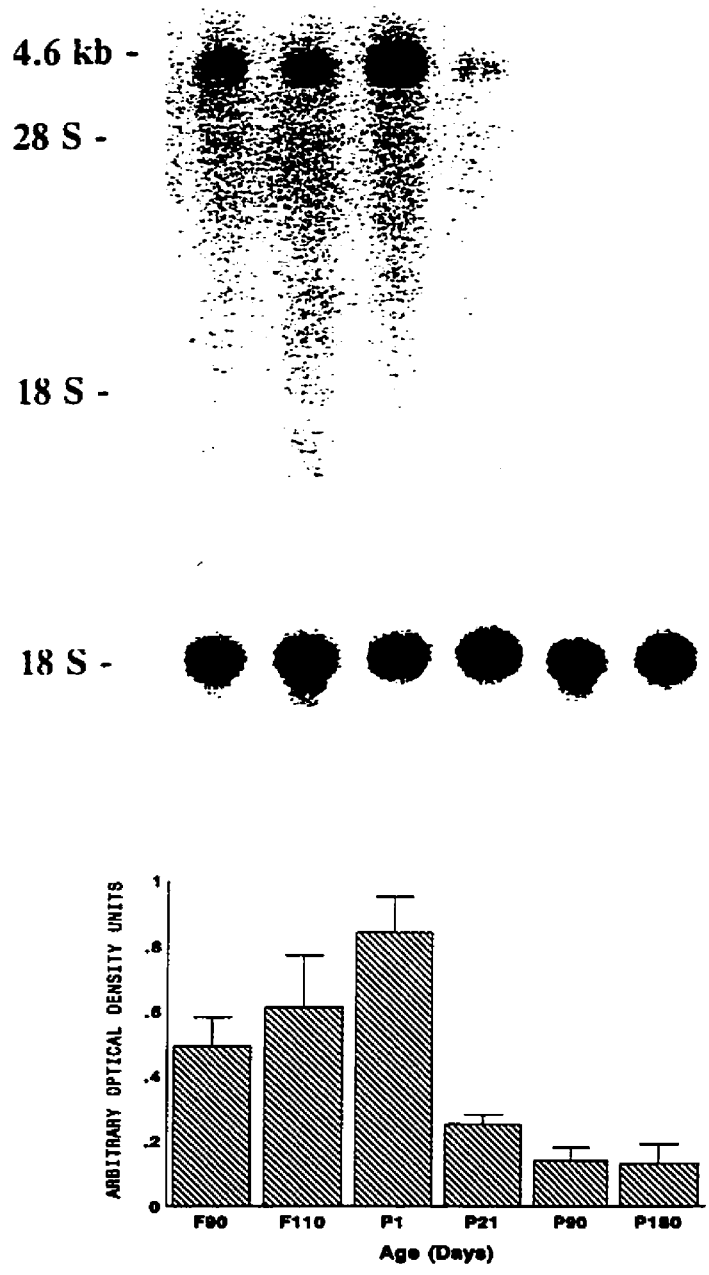
Age (Days)

Figure 6.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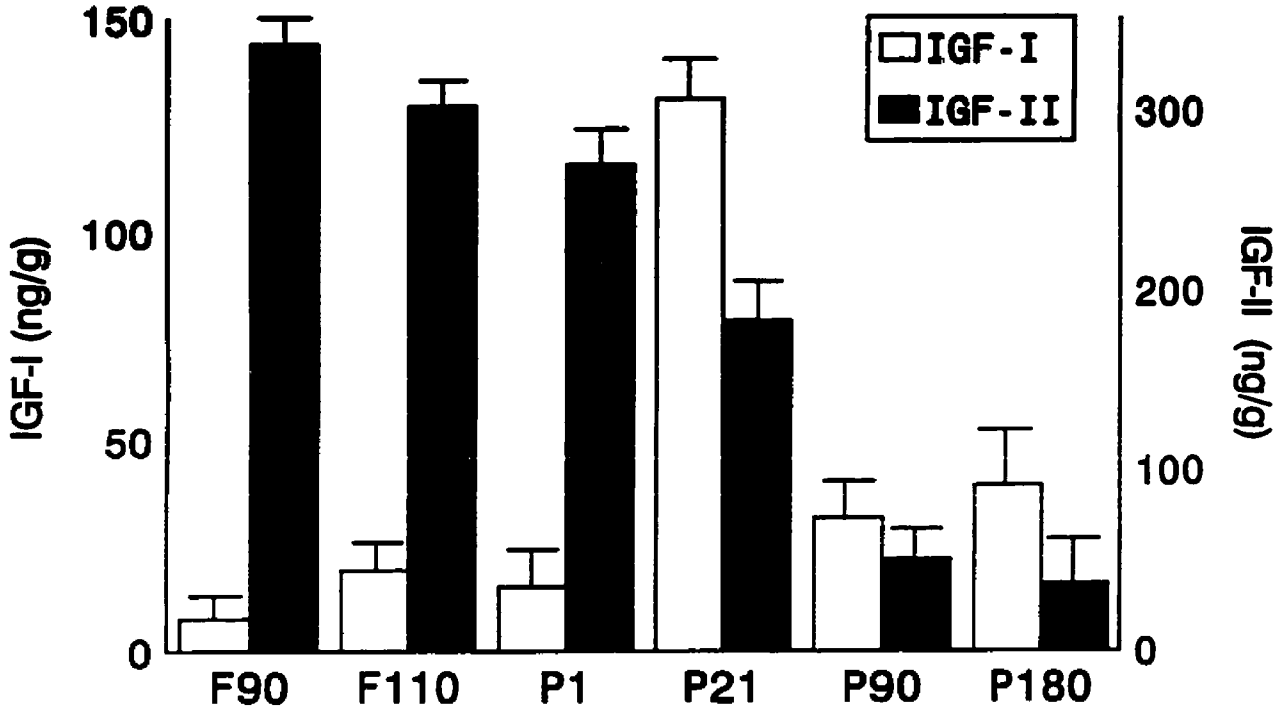
Age (Days)

Figure 7.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IGF-I
IGF-II
300
200
100
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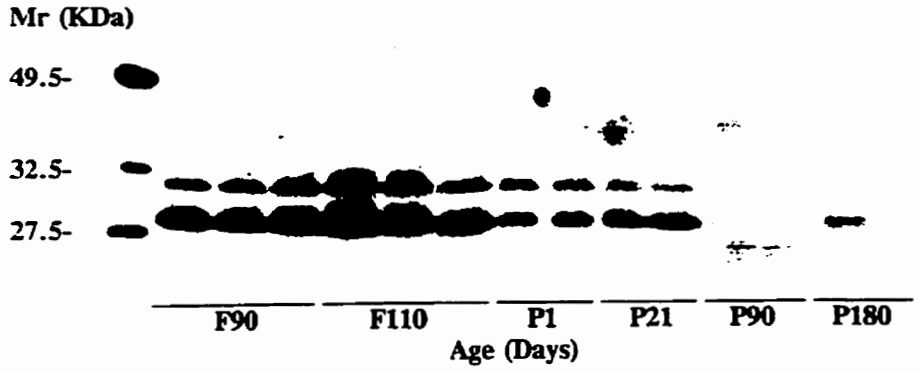
IGF-II (ng/g)
F90
F110
P1
P21
P90
P180

Figure 8.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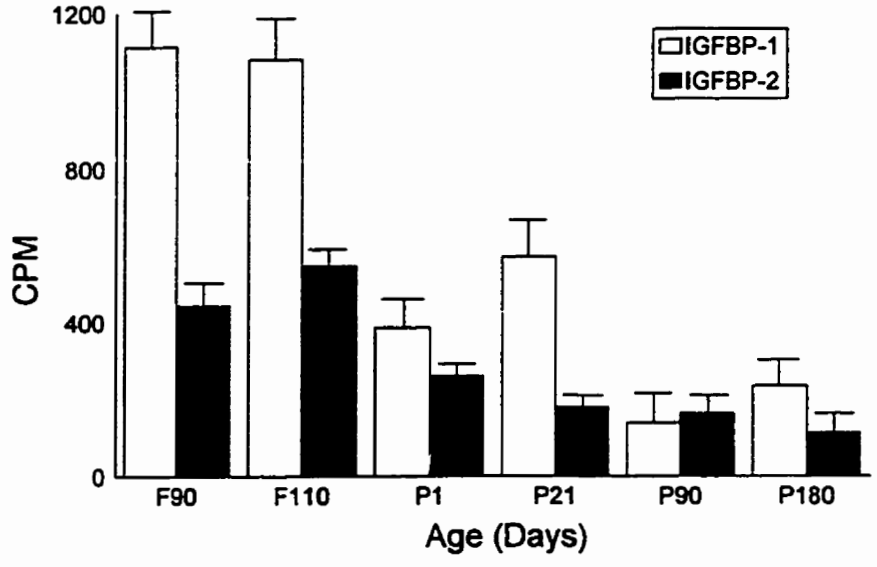
27.5-
F90
F110
P1
P21
P90
P180
Age (Days)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800
400
0
CPM
IGFBP-1
IGFBP-2
F90
F110
P1
P21
P90
P180
Age (Days)

Figure 9.

2.2 M. Peng, G. Pelletier, M.-F. Palin, S. Véronneau, D. LeBel and T. Abribat. ONTOGENY OF IGFs AND IGFBPs mRNA LEVELS AND TISSUE CONCENTRATIONS IN LIVER, KIDNEY AND SKELETAL MUSCLE OF PIG. Growth Dev. Aging, 1996. 60:171-187.

2.2.1 Contribution to manuscript 2.2

My contribution to this manuscript was to (1) plan and write the project outline, (2) prepare and organize sample collection, (3) extract total RNA and carry out Northern analysis, (4) provide data to the statistician, (5) prepare figures and tables and write manuscript. Dr. T. Abribat's laboratory determined IGFs and IGFBPs concentrations by RIA and Western blot analysis respectively. We have also got technical assistance from Dr. M-F. Palin and Mr. S. Véronneau for probe preparation. The research project and the manuscript preparation were directed by Drs. G. Pelletier and D. LeBel.

2.2.2 Manuscript of 2.2

*Growth, Development & Aging*, 1996, 60, 171-187



# Ontogeny of IGFs and IGFBPs mRNA Levels and Tissue Concentrations in Liver, Kidney and Skeletal Muscle of Pig

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**ABSTRACT:** As far as we know, there is no available information about ontogenic changes of tissue concentrations of IGF-I and II and IGFBPs in large mammals. Serum, liver and kidney levels of IGFs and IGFBPs were examined in fetuses at 90 and 110 days of gestation and in pigs at 1d, 3 wk, 3 mo and 6 mo of age. Ontogeny of mRNA levels of IGFs, IGF type I and type II receptors (IGFI-R and IGFII-R), IGFBP-1 and -3 (IGFBPs) and growth hormone receptor (GHR) were also examined by Northern blot analysis in liver, kidney and skeletal muscle of pig. Serum IGF-I, IGF-II and IGFBP-3 concentrations were low during the fetal life and increased after birth. The highest level of IGF-II mRNA was found in fetuses for all studied tissues. In the liver, IGF-I mRNA level and its protein content peaked at 3 wk of age. The highest IGF-II concentration was found at 1d and 3 wk of age. The IGFII-R mRNA remained at a constant level during the whole development period. The most abundant IGFBP-1 mRNA and its protein content were found at birth. The level of IGFBP-2 was high during fetal and early postnatal life. The IGFBP-3 content was relatively low in fetuses and reached the highest level after 3 wk of age. In the kidney, IGFs, IGFBP-3, IGFI-R and IGFII-R as well as GHR mRNA levels were relatively high during the fetal and early postnatal life. The IGFs concentrations were the highest in newborns. In the skeletal muscle, IGFs, IGFBP-3 and IGFI-R mRNA levels decreased with advancing age. During the postnatal life, the high IGFs concentrations in the liver and the kidney correspond to fast growth periods of these organs.

**KEY WORDS:** IGFs, IGFBP, Tissue, Age

## INTRODUCTION

Insulin-like growth factor -I and -II (IGF-I and -II or IGFs) are important regulators of cell proliferation, differentiation and metabolism (Jones and Clemmons 1995). Mouse embryos carrying a null mutation of the gene encoding IGF-I, IGF-II and type I receptor (IGFI-R) showed growth deficiency (Baker *et al.*, 1993, Liu *et al.*, 1993). The role of IGF-I as a mediator of growth hormone action and as a

local autocrine/paracrine stimulator of cell growth for a variety of cell types is widely accepted (LeRoith and Roberts 1991). However, very few studies are available regarding tissue concentrations and mRNA levels of IGFs in large mammals such as pigs during development. The pig is a good animal model because of its anatomical and physiological similarities with human.

IGFs present in serum and other extracellular fluids are associated with specific IGF binding proteins (IGFBPs), which seem to control the bioavailability of the IGFs and also modulate binding of IGFs to their target cell receptors (Clemmons 1991). So far six IGFBPs designated IGFBP-1 to -6 have been identified and their cDNAs cloned (Shimasaki and Ling 1991). IGFs and IGFBPs mRNAs were detected in various rat (Brown *et al.*, 1986, Albiston and Herington 1992) and pig tissues (Lee *et al.*, 1993). It was reported that IGFs and IGFBPs were synthesized in many tissues and released from many cell lines in culture (Sara and Hall 1990; Rutanen and Pekonen 1990). The IGF-I and -II peptides have also been detected in the liver, kidney and muscle of pig fetuses (Hausman *et al.*, 1991). However, as far as we know no information is available on ontogeny of IGFs concentration in various tissue of pig.

It is well known that growth hormone (GH) plays a key role in the regulation of mammalian growth and that IGF-I is the major mediator of GH's actions on somatic growth (LeRoith and Roberts 1991). Messenger RNA of GH receptor (GHR) and GHR protein have been identified in rat fetal tissues (Garcia-Aragon *et al.*, 1992; Walker *et al.*, 1992). In rabbit, man (Leung *et al.*, 1987) and pig (Lee *et al.*, 1993), it is believed that GH binding protein (GHBP) results from a proteolytic cleavage of GHR. An *in situ* hybridization study has demonstrated a distinct distribution of GHR/BP and IGF-I mRNAs in developing rat fetuses with coordinate expression at several mesenchymal sites, such as dermis and gut (Edmondson *et al.*, 1995). In the pig, GHR specific binding and GHR mRNA level were found in skeletal muscle from 75 days of gestation until the adult stage with no clear age-related changes, whereas GHR in the liver increased with advancing age (Schnoebelen-Combes *et al.*, 1996). This suggests that GH is involved in skeletal muscle development and local accumulation of GHR mRNA is tissue specifically regulated.

The objectives of the present study were to examine tissue concentrations and mRNA levels of IGFs, IGFBPs and GHR the growth of liver and kidney and their relationships during pig development.

## MATERIAL AND METHODS

### *Animals*

Yorkshire purebred fetuses from 90 (F90) and 110 (F110) days of gestation (normal gestation time is 114 days) and pigs of 1d (P1), 3 wk (P21), 3 mo (P90) and 6 mo (P180) of age were used. Age of fetuses was determined according to the sows mating date. At each age two males and two females were obtained from one litter of three different sows to make the calculations on biochemical parameters. Therefore, we used 12 animals at each age including 6 males and 6 females to check the effect of sex x age interaction. Pigs including sows, with the exception of fetuses were fasted for 16 h before slaughtering. Animals were slaughtered (stunning and exsanguination) according to recommended code of practice for the care and handling of farm animals, pigs (Agriculture and Agri-Food Canada, 1993). After laparotomy, the uterus was gradually opened and the fetuses were removed. Immediately after exsanguination, liver and kidney were removed by dissection and weighed. A sample of skeletal muscle from the biceps femoris was also taken.

### *Preparation of blood and tissue samples and protein, RNA and DNA measurements*

Blood samples were obtained from the jugular vein and left at room temperature for 4h, then at 4°C overnight and after centrifugation the serum samples were stored at -20°C. Tissue samples of liver, kidney and skeletal muscle were frozen in liquid nitrogen and stored at -80°C until used for Northern blot analysis and radioimmunoassay (RIA) measurements. Fresh tissue samples were used for total protein, RNA and DNA determinations according to the methods described previously (de Passillé *et al.*, 1989).

### *RIA*

Serum and tissue IGF-I (Abribat *et al.*, 1990) and -II (Lee *et al.*, 1991 b) concentrations were quanti-

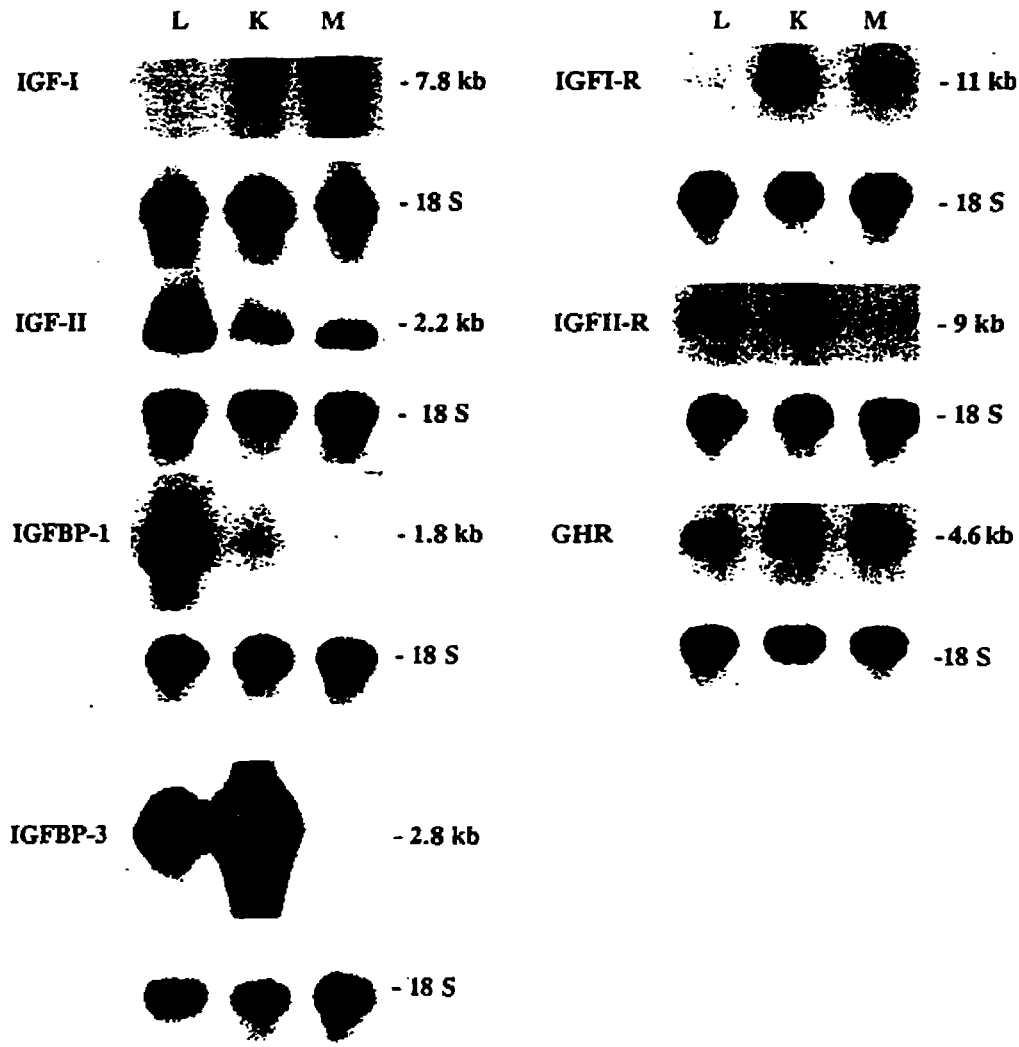


**Figure. 1:** Representive Northern blot of IGF-I, -II, IGFI-R, IGFII-R, IGFBP-1, IGFBP-3 and GHR mRNAs in various fetal tissues (L: liver, K: kidney, M: muscle). Total RNA was applied to 1% agarose-formaldehyde gel followed by blot hybridization using a IGF-I and IGFI-R cDNA probes (at 65°C) and IGF-II, IGFII-R, IGFBP-1, IGFBP-3 and GHR RNA probes (at 65°C). Filters were washed twice at 60°C for cDNA probes and 85°C for RNA probes with 0.1( SSC/0.1% SDS for 30 min. Autoradiography proceeded for approximately two weeks for IGF-I, IGFBP-1, IGFI-R, IGFII-R and GHR, 2h for IGF-II and two days for IGFBP-3 respectively at -80°C. The sizes of the transcripts were estimated according to the position of 18S and 28S. The blots were reprobed with a 18S ribosomal $^{32}$P-labelled probe as a control to quantitate RNA loading and transfer.

fied by RIA using methodologies previously described. In our assay conditions, ED50s from standard curves were 60 ± 8 pg/tube and 86 ± 10 pg/tube for IGF-I and -II, respectively (mean ± SD of 10 determinations). Intra and inter assay coefficients of variation were 4 and 13% and 3 and 12% for IGF-I and IGF-II, respectively.

*Western ligand blotting for IGFBPs*

Serum, liver and kidney acid extracts were subjected to Western ligand blot analysis as described by Hossenlopp *et al.*, (1986). Initial experiments in our laboratory showed that neutralized acid extracts gave similar results to extracts performed in neutral conditions. Therefore, the 40,000 x g supernatants of the formic acid extracts were neutralized with 2 M Tris buffer, pH 8.5, then loaded onto a 12% SDS polyacrylamide gel and electrophoresed. Proteins were then transferred to nitrocellulose (BioRad, Richmond, CA) at 15 V overnight in a Transblot Cell (BioRad, Richmond, CA). The blotted membranes were washed and then incubated with $^{125}$I-IGF-I ($1.4x10^5$ cpm/ml) in 30 ml tris buffer saline (TBS), 0.1% bovine serum albumin (BSA), over night at 4°C. Membranes were washed twice for 15 min in TBS, 0.1 % Tween 20, three times for 15 min in TBS, dried X ray films were exposed in the presence of intensifying screen for 3 days at -80°C. For quantitative analysis, bands of the membranes corresponding to those on autoradiograms were cut and counted in a gamma counter. For each individual IGFBP, data were expressed as cpm.

*RNA preparation*

Total RNAs were extracted according to the method described by Chirgwin *et al.*, (1979). Tissues were homogenized in 4 M guanidinium thiocyanate using a polytron homogenizer. Supernatants were used for precipitation of RNA in 100 % ethanol and 2 M potassium acetate at -20°C. After centrifugation, the RNA pellets were dissolved in 7 M guanidine hydrochloride. Then, RNA were precipitated and quantitated by measuring its absorbance at 260 nm.

*Preparation of probes*

A human IGF-I cDNA which is a 659 bp cDNA EcoR1 fragment (Jansen *et al.*, 1983) and a human IGF-IR cDNA (Ullrich *et al.*, 1986) corresponding to a 700 bp cDNA EcoR1 fragment were used. The cDNA probes were labelled with [$^{32}$P]dCTP (3000 Ci/mmol; Amersham, Canada Limited, Oakville, ON) by random priming using a commercial kit (Megaprime DNA labelling System, Amersham Canada Limited, Oakville, ON).

Rat IGF-II, human IGFBP-1, porcine IGFBP-3 and human IGF-II receptor complementary RNA (cRNA) probes were transcribed from a 720 base pairs (bp) *Bam*H1 cDNA fragment (Ueno *et al.*, 1988), a 1443 bp *Eco*R1 cDNA fragment (Julkunen *et al.*, 1988), a 595 bp *Eco*R1-*Sma*I cDNA fragment (Shimasaki *et al.*, 1990) and a 695 bp *KpnI-Hind* III fragment (Morgan *et al.*, 1987), respectively. The cDNA fragments were inserted into the pBluescript KSII+ plasmid vector. The inserts were sequenced using a T7Sequencing™ Kit (Pharmacia Biotech Inc, Piscataway, NJ) in order to check the orientation of the cDNAs. The templates were linearized with *Eco*R1 for IGF-II, *Hind*III for IGFBP-1, *Sam*1 for IGFBP-3 and *Kpn*I for IGFII-R and then used in a T7 transcription Riboprobe System (Promega, Madison, WI).

The GHR probe was prepared by RT-PCR. The GHR primers were designed according to known regions of the pig GHR cDNA sequence (Cioffi *et al.*, 1990). The upstream primer (5'- ATCCCGCGGAACCACCTCCCAATGCAGATGTTC-3') is identical to nucleotide 545-568, while the downstream primer (5'-ATCTCTAGATGGCGCTAGACGTGATTCAACCTC-3') represents the reverse complement of nucleotides 1669-1692. This primer pair predicts a 1166 bp fragment. A PCR amplified cDNA fragment of the predicted length was inserted into the pBluescript KSII+ plasmid vector. The authenticity of the cDNA clone was verified by sequencing using a T7Sequencing™ kit (Pharmacia Biotech Inc., Piscataway, NJ). The template was linearized with *Eco*RV and then used in a T7 transcription Riboprobe System (Promega, Madison, WI) to synthesis the cRNA probe.

*Northern blot analysis*

The procedure for Northern blot was described by Lehrach *et al.*, (1977) with some modifications. Briefly, twenty µg of total RNA was fractionated in a 1% agarose gel containing 2.2 M formaldehyde before transfer to nylon membranes (Nytran, Schleich-

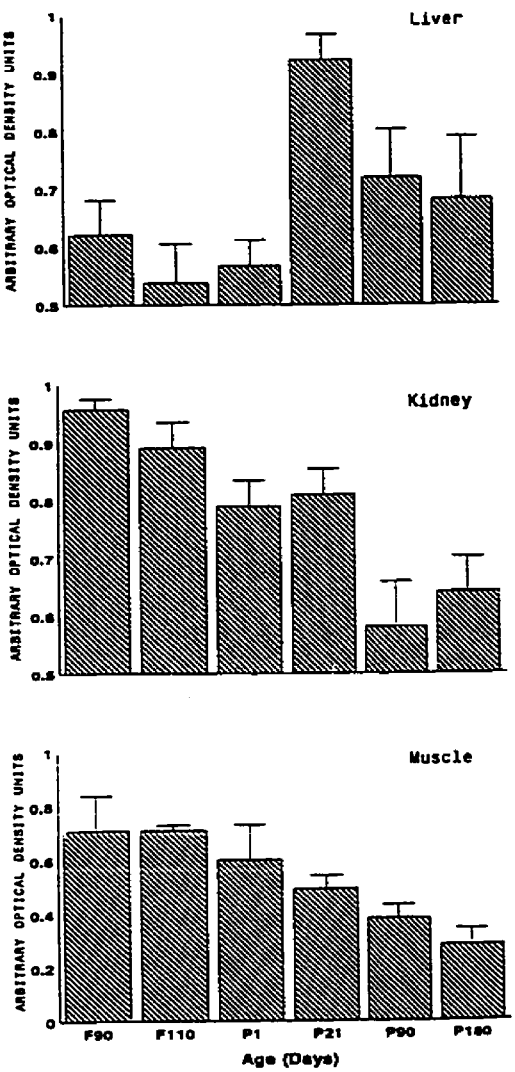


Figure 2: Northern analysis of IGF-I mRNA levels (7.8kb transcript) in various tissues of pig during development (F: fetus, P: postnatal). The densitometric scanning of the autoradiograms was expressed in arbitrary optical unit as a ratio of 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Date at each age represent the mean values ± SE obtained from four observations (one male and one female per litter from two different litters).

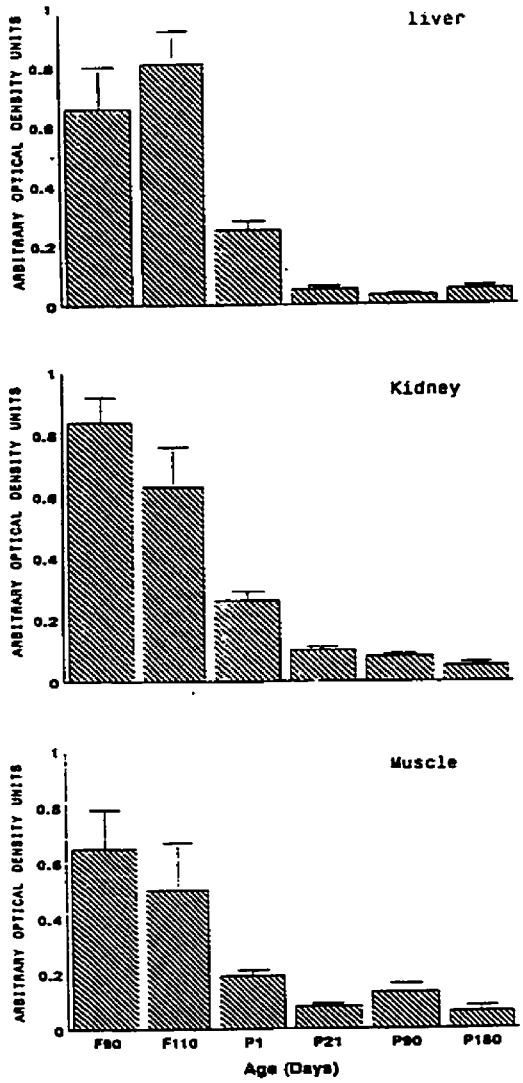


Figure 3: Northern analysis of IGF-II mRNA levels in various tissues of pig during development (F: fetus, P: postnatal). The densitometric scanning of the autoradiograms was expressed in arbitrary optical unit as a ratio of 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Date at each age represented the mean values ± SE obtain from four observations (one male and one female per litter obtain from two different litters).

er and Schuell, Keene, NH). For RNA probes, membranes were prehybridized for 4 h in a solution of 50% formamide, 5 x SSC, 50 mM Hepes pH 6.8, 2 mM EDTA pH 8.0, 5 x Denhardt's, 1% SDS and 200 µg/ml of salmon sperm DNA at 65°C. They were then hybridized in the same solution overnight at 65°C with cRNA riboprobes (rat IGF-II, human IGFII-R, human IGFBP-1, porcine IGFBP-3 and pig GHR) which were labelled with [$^{32}$P]-labelled dUTP. For DNA probes, membranes were prehybridized for 15 min in a solution (Rapid-hyb buffer, Amersham Canada Limited, Oakville, ON) at 65°C, and then hybridized in the same solution for 2 h at 65°C with cDNA probes (human IGF-I and human IGF-I receptor) labelled with [$^{32}$P]-labelled dCTP. Membranes were then washed with 0.1 x SSC/ 0.1% SDS twice, 30 min at 85°C for RNA probes and at 60°C for cDNA probes and exposed to Kodak films (Eastman Kodak Company, Rochester, NY) with intensifying screen at -80°C. The blots were reprobed with 18S ribosomal $^{32}$P-labelled cDNA probe as a control to quantitate RNA loading and transfer. The size of RNA transcripts was evaluated according to the position of 18S and 28S rRNAs or RNA ladder.

Autoradiograms were scanned using a densitometer (BIO-RAD Imaging Densitometer Model GS-670, Biorad Laboratories Led., Mississauga, ON). The relative density of the transcript is expressed as arbitrary optical unit. To correct for the possible differences in loading of total RNA in Northern blots, a ratio of the relative density of each specific transcript of growth factors with the relative density of the 18S ribosomal RNA band was calculated before statistical analysis was performed. For Northern blot, we used four animals per age including one male and one female per litter to make one membrane. For each probe we used two membranes.

*Statistical analysis*

We ran an analysis of variance (SAS, 1985) on parameters using age of pig as main effect, sow within each age as first error term, sex of piglets as a split effect and age x sex interaction. The different ages were compared using *a priori test* comparisons. The following comparisons were used: (1) fetuses (F90 + F110) vs suckling piglets (P1 + P21), (2) suckling (P1 + P21) vs postweaning (P90 + P180) pigs, (3) within fetal ages: F90 vs F110, (4) within suckling ages: P1 vs P21, (5) within postweaning ages: P90 vs P180. For the Northern blot analysis, the same model as the previous one was used except that the membrane effect was tested instead of the sow effect. Data were considered significantly different if probability value ($P$) ≤0.05.

## RESULTS

*IGF-I, IGF-II, IGFBP-1, IGFBP -3, IGFI-R, IGF-IIR and GHR mRNA levels*

An IGF-I mRNA transcript of approximately 7.8 kilobases (kb) was detected in all studied tissues (Fig. 1). In 110 day-old fetuses skeletal muscle exhibited the highest level of IGF-I mRNA, while liver had the lowest one (Fig. 1). In the liver, the IGF-I mRNA level was the highest (P1 vs P21: $P = 0.05$) at 3 wk of age, with no obvious change at any other developmental stages (Fig. 2). In the kidney, the highest level of IGF-I mRNA was observed during fetal life and decreased ($P = 0.02$) after birth (Fig. 2) and a further decrease ($P < 0.05$) occurred at postweaning age (P90 to P180). In the skeletal muscle, IGF-I mRNA level decreased ($P < 0.05$) with age (Fig. 2). A single IGF-II mRNA transcript of approximately 2.2 kb was found in every studied tissue (Fig. 1). The highest level of IGF-II mRNA was observed during the fetal life and decreased significantly ($P < 0.05$) after birth in every tissue (Fig. 3). The liver IGF-II mRNA level of male was higher ($P = 0.005$) than female (1.00 ± 0.04 vs 0.62 ± 0.04) during the fetal F110 as indicated by a significant age and sex interaction effect ($P = 0.02$).

An IGFBP-1 mRNA transcript of 1.8 kb was observed in the liver and kidney but not in the skeletal muscle (Fig.1). In the liver, IGFBP-1 mRNA reached the highest level at P1 (Fig. 4) while at the same period it was very low in the kidney (data not shown). A single 2.8 kb mRNA transcript for IGFBP-3 was detected in all studied tissues (Fig. 1). The IGFBP-3 mRNA level in the kidney was higher than that of liver and skeletal muscle. In the liver, the level of IGFBP-3 mRNA was low in F90, then increased ($P = 0.05$) in F110 and remained at this level until P180 (Fig. 5). In the kidney, the maximum level of IGFBP-3 mRNA occurred during the fetal and early postnatal life and decreased ($P = 0.05$) during the postweaning period (Fig. 5). In the skeletal muscle, high levels of IGFBP-3 mRNA were observed in fetuses and newborns and decreased (fetuses vs suck-

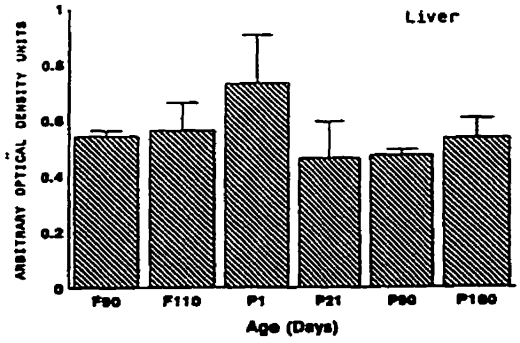


**Figure 4:** Northern analysis of the IGFBP-1 mRNA level in pig liver during development (F: fetus, P: postnatal). The densitometric scanning of the autoradiogram was expressed in arbitrary optical unit as a ratio of 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Date at each age represent the mean values ± SE obtained from four observations (one male and one female per litter from two different litters).

ling: $P = 0.003$) thereafter (Fig. 5).

IGFI-R mRNA was expressed as a single 11 kb transcript in kidney and skeletal muscle, but it was not detectable in the liver (Fig. 1). The IGFI-R mRNA level was higher in the kidney than that of muscle. In kidney and skeletal muscle, IGFI-R mRNA was relatively abundant in the fetal life and decreased ($P < 0.05$) after birth (Fig. 6). In the kidney, IGFI-R mRNA was more ($P = 0.005$) abundant in male than female during fetal (F90; 0.67 ± 0.06 vs 0.74 ± 0.03) and adult (P180; 0.10 ± 0.00 vs 0.34 ± 0.08) life. An IGFII-R mRNA transcript of approximately 9 kb was found in liver and kidney, but not detected in the skeletal muscle (Fig. 1). In the liver, the IGFII-R mRNA level remained high during the whole development period (Fig. 7). In the kidney, IGFII-R mRNA levels were the highest in fetal and neonatal piglets, then decreased (fetuses vs suckling: $P = 0.01$) from 3 wk of age (Fig. 7). One transcript of approximately 4.6 kb for GHR mRNA was detected in all examined tissues (Fig. 1). In the liver, GHR mRNA levels were low during fetal life and increased (fetuses vs suckling: $P = 0.01$) after birth (Fig. 8). In the kidney, GHR mRNA levels were relatively high during fetal and suckling period, then decreased ($P = 0.01$) in postweaning period (Fig. 8).

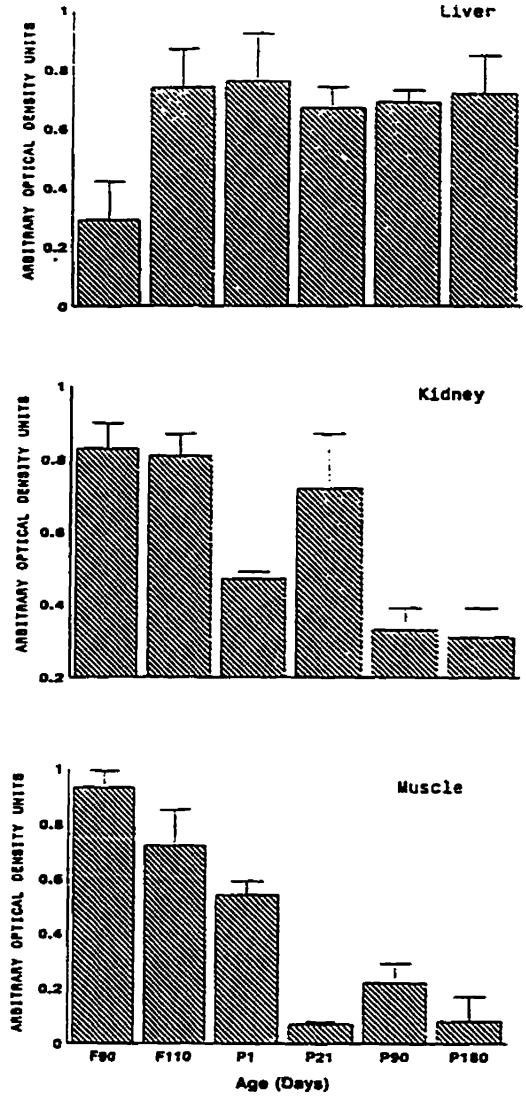


**Figure 5:** Northern analysis of IGFBP-3 mRNA levels in various tissues of pig during development (F: fetus, P: postnatal). The densitometric scanning of the autoradiograms was expressed in arbitrary optical unit as a ratio of 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Date at each age represent the mean values ± SE obtained from four observations (one male and one female per litter from two different litters).

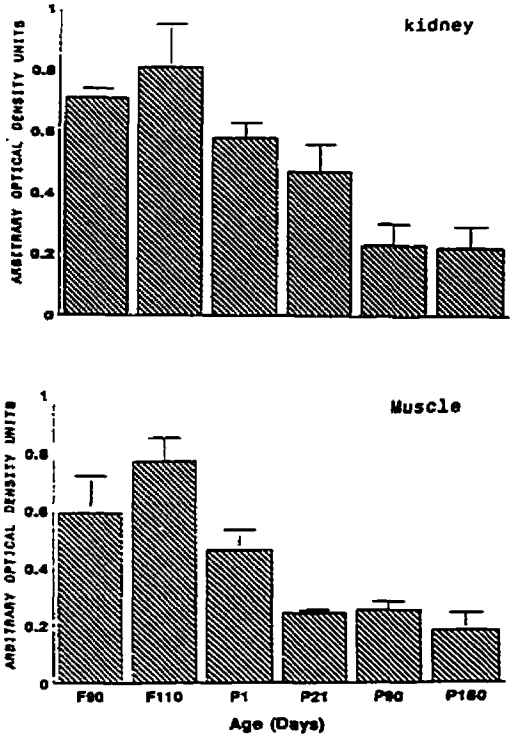


Figure 6: Northern analysis of IGFI-R mRNA levels in various tissues of pig during development (F: fetus, P: postnatal). The densitometric scanning of the autoradiograms was expressed in arbitrary optical unit as a ratio of 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Date at each age represent the mean values ± SE obtained from four observations (one male and one female per litter from two different litters).

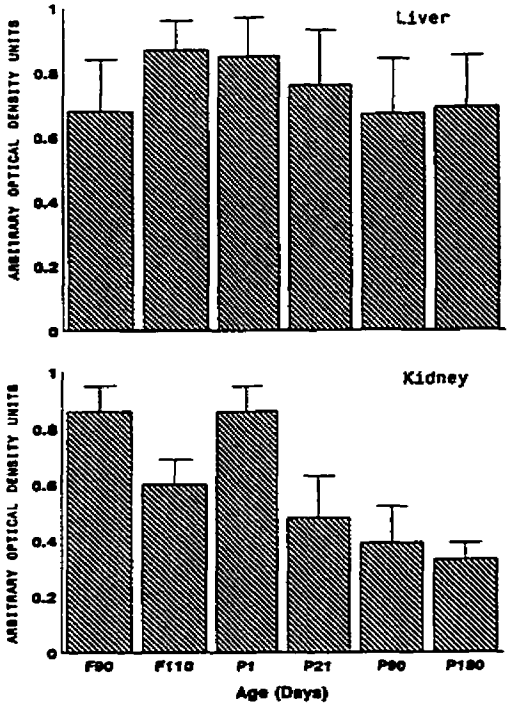


Figure 7: Northern analysis of IGFII-R mRNA levels in various tissues of pig during development (F: fetus, P: postnatal). The densitometric scanning of the autoradiograms was expressed in arbitrary optical unit as a ratio of 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Date at each age represent the mean values ± SE obtained from four observations (one male and one female per litter from two different litters).

There was no significant changes of GHR mRNA level in skeletal muscle during the whole developmental period examined.

*Serum and tissue IGFs and IGFBPs concentrations*

Serum IGF-I concentration was low during the fetal life and increased ($P < 0.05$) with advancing age (Fig. 9A). The IGF-II serum concentration increased markedly at 6 mo of age. Serum IGFBP-1 and -4 levels were relatively low in fetuses, peaked at birth and decreased (P1 vs P21: $P < 0.05$) at P21 (Fig. 9B and C). Levels of IGFBP-2 were relatively high in fetal 90 days and newborn animals while at the same time IGFBP-3 levels were low with no obvious changes after P21. Serum IGFBP-3 content was more abundant than that of the other IGFBPs during postnatal life.

In the liver, the highest IGF-I and -II concentrations were observed at P21 and suckling period (P1 and P21), respectively (fetuses vs suckling, suckling vs postweaning: $P < 0.05$, Fig. 10A). Liver IGFBP-1 and -4 were higher ($P < 0.05$) between P1 and P21 than that of the other developmental ages ( Fig. 10B and C). The IGFBP-2 level was higher ($P = 0.05$) during fetal and early postnatal life than the postweaning period. The level of IGFBP-3 was relatively low in fetuses and newborns, increased (P1 vs P21: $P = 0.02$) at P21.

In the kidney, the highest concentrations of both IGF-I and -II occurred at birth ($P < 0.05$, Fig. 11A). The IGFBP-1 and -2 levels were abundant from F90 to P21 and low ($P < 0.05$) at P90 and P180 (Fig. 11B and C). The highest level of IGFBP-3 and 4 was observed at P21 and P1 respectively ($P < 0.01$).

*Growth of the liver and the kidney*

The growth of the liver was high in fetuses up to 3 wk of age as measured by the ratio of liver weight / body weight (Table 1). However, a marked decrease (sucking vs postweaning: $P < 0.01$) of the liver weight to body weight ratio occurred at P180 as a result of a faster body growth relative to liver growth from P90 to P180. The highest ratio of total liver DNA / body weight was observed in F90 then decreased (fetuses vs suckling: $P < 0.001$) with advancing age. During the suckling period, the ratio of DNA / body weight was higher ($P = 0.001$) at P21 than that at P1. The highest levels of liver weight to DNA and total RNA to DNA ratios were reached in newborns (fetuses vs suckling: $P < 0.001$).

In the kidney, organ weight / body weight and DNA / body weight ratios were relatively high ($P < 0.05$) during fetal and early postnatal life, and then decreased ($P < 0.001$) with advancing age (Table 2). In suckling piglets, ratios of kidney weight and DNA / body weight were higher ($P < 0.001$) at P1 than that of P21. Kidney weight to DNA, total RNA and protein to DNA were the lowest during fetal life and increased (fetuses vs suckling: $P < 0.001$) with developmental stages.

*Discussion*

This study is the first attempt to look at the relationships between mRNA levels, tissue and serum concentrations of IGFs and IGFBPs as well as organ

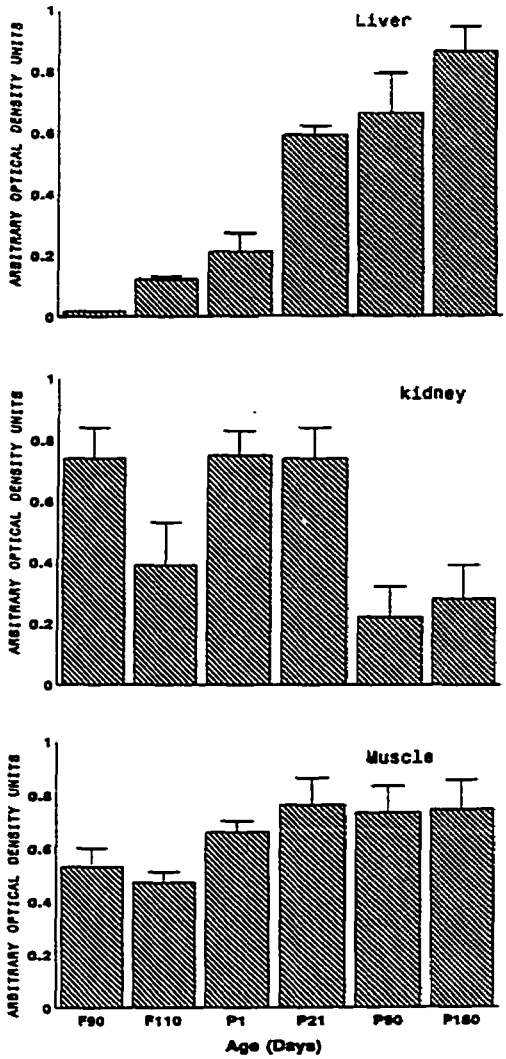


**Figure 8:** Northern analysis of GHR mRNA levels in various tissues of pig during development (F: fetus, P: postnatal). The densitometric scanning of the autoradiograms was expressed in arbitrary optical unit as a ratio of 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Date at each age represent the mean values ± SE obtained from four observations (one male and one female per litter from two different litters).

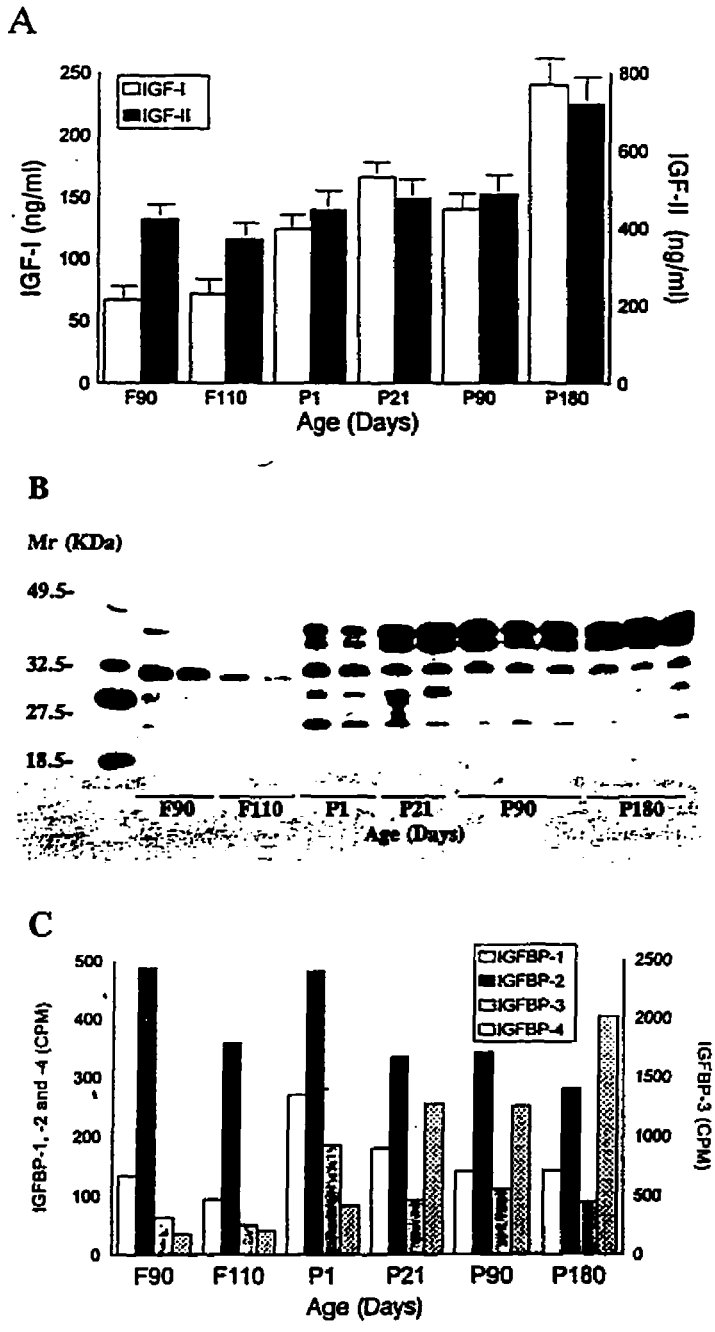


**Figure 9:** Effect of age on serum IGF-I, -II and IGFBPs concentrations in pig (F: fetus, P: postnatal). A: IGF-I and IGF-II concentrations were determined by RIA. Values are means of 6 to 12 observations (at least one male and one female per litter from three different litters were used). B: Representative Western blotting of IGFBPs in serum. Serum was separated on a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis under non-reducing conditions and transferred onto nitrocellulose membrane. Blots were probed with $^{125}$I-labelled IGF-I and autoradiographed at -80°C for 72 h. Bands of the membrane corresponding to those on the autoradiograms were counted in a gamma counter and expressed in cpm. C: Average values of IGFBPs from three Western blots are described in panel B. (IGFBP-1=27.5kDa; IGFBP-2=32.5kDa; IGFBP-3=39.0 kDa; IGFBP-4=26.0 kDa)

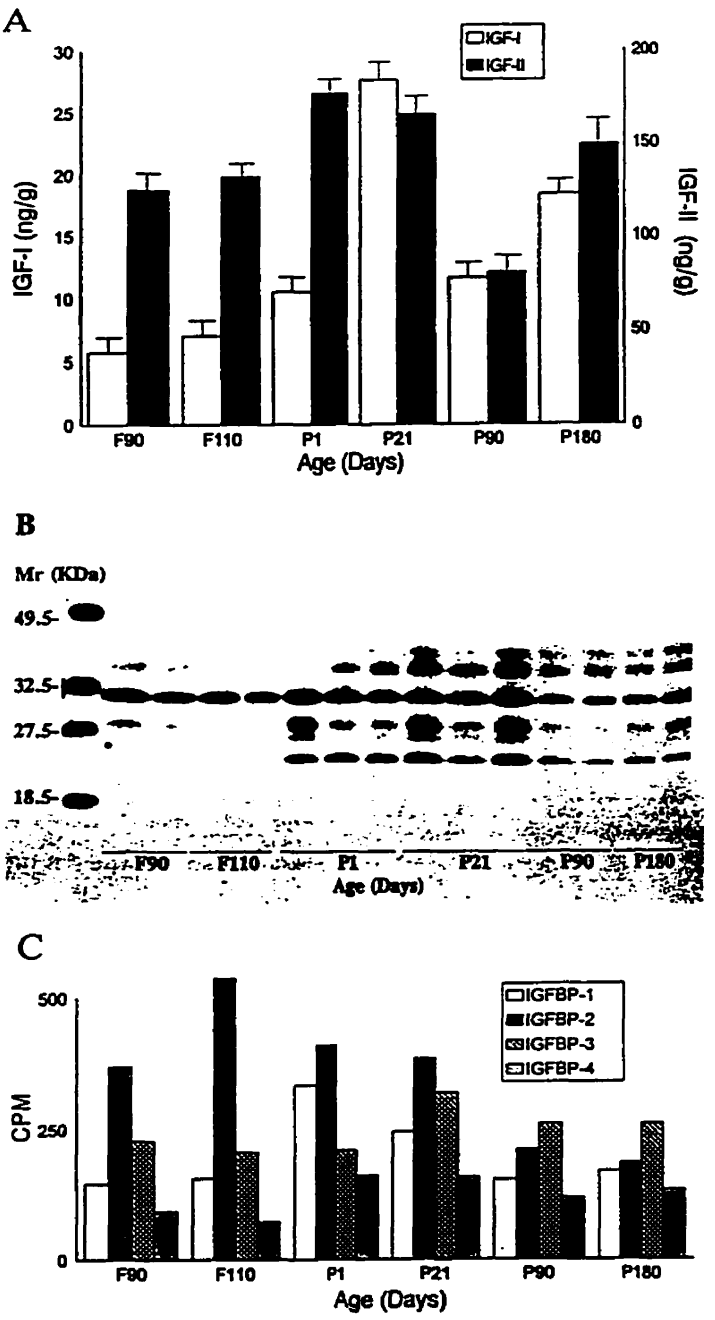


**Figure 10:** Effect of age on IGF-I, -II and IGFBPs concentrations in liver of pig (F: fetus, P: postnatal). A: IGF-I and IGF-II concentrations were determined by RIA on liver homogenates prepared by homogenization of 500 mg to 1g of tissue in a 1 g to 4 ml ratio wt/vol of 3.3 M formic acid solution to dissociate the IGFBPs complex. Values are means of 6 to 12 observations (at least one male and one female per litter from three different litters were used). B: Representative Western blotting of IGFBPs in liver. Liver homogenates were separated on a 12 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis under non-reducing conditions and transferred onto nitrocellulose membrane. Blots were probed with $^{125}$I-labelled IGF-I and autoradiographed at -80°C for 72 h. Bands of the membrane corresponding to those on the autoradiograms were counted in a gamma counter and expressed in cpm. C: Average values of IGFBPs from three Western blots are described in panel B. (IGFBP-1=27.5kDa; IGFBP-2=32.5kDa; IGFBP-3=39.0 kDa; IGFBP-4=26.0 kDa)

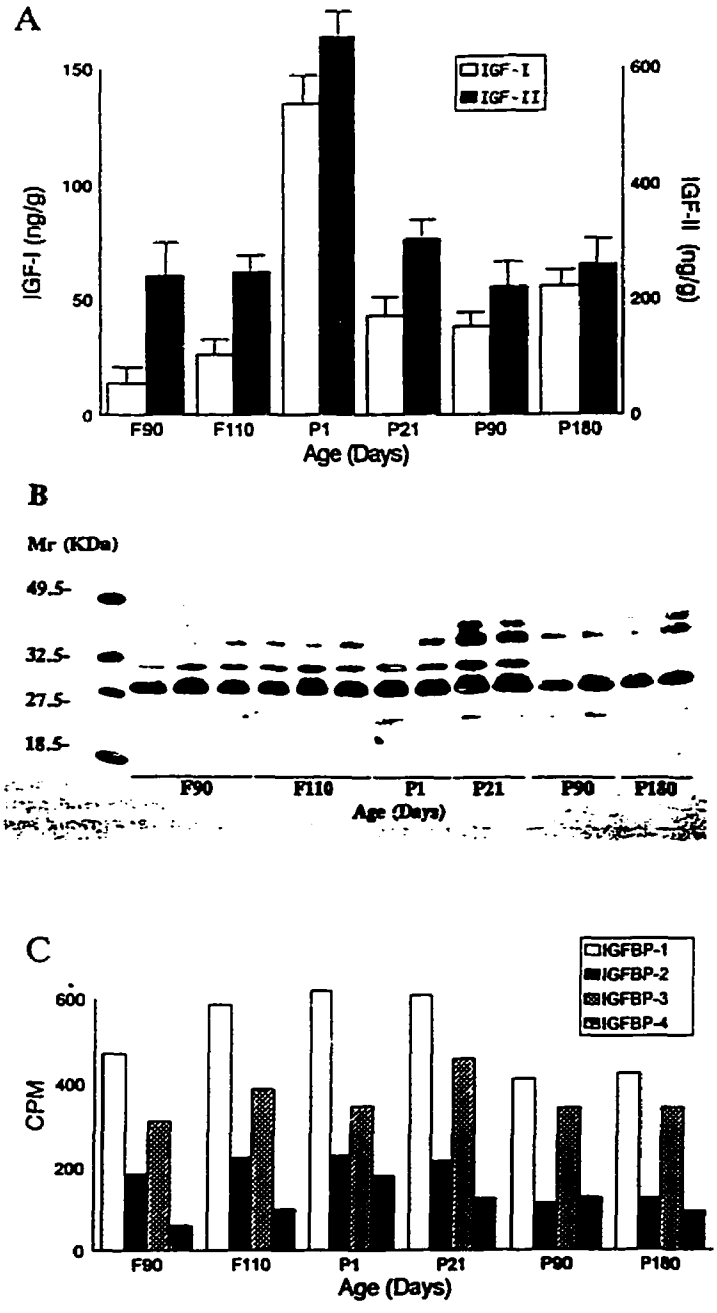


**Figure 11:** Effect of age on IGF-I, -II and IGFBPs concentrations in kidney of pig (F: fetus, P: postnatal). A: IGF-I and IGF-II concentrations were determined by RIA on kidney homogenates prepared as described in Fig. 2 panel A. Values are means of 6 to 12 observations (at least one male and one female per litter from three different litters were used). B: Representative Western blotting of IGFBPs in kidney. Kidney homogenates were separated on a 12 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis under non-reducing conditions and transferred onto nitrocellulose membrane. Blots were probed with $^{125}$I-labelled IGF-I and autoradiographed at - 80°C for 72 h. Bands of the membrane corresponding to those on the autoradiograms were counted in a gamma counter and expressed in cpm. C: Average values of IGFBPs from three Western blots are described in panel B. (IGFBP-1=27.5kDa; IGFBP-2=32.5kDa; IGFBP-3=39.0 kDa; IGFBP-4=26.0 kDa)

TABLE 1
Effect of age on liver weight and total DNA to body weight ratios and liver weight, total RNA and protein to DNA ratios in the liver of pig

| Age (Days) | Liver Weight/ Body Weight (g/kg) | DNA/ Body Weight (mg/kg) | Liver Weight/ DNA (g/mg) | RNA/ DNA (mg/mg) | Protein/ DNA (mg/mg) |
|---|---|---|---|---|---|
| F90 | 28.5 ± 1.3 | 306.4 ± 17.7 | 0.09 ± 0.00 | 0.64 ± 0.02 | 7.9 ± 0.16 |
| F110 | 29.0 ± 1.6 | 137.9 ± 11.9 | 0.22 ± 0.01 | 1.05 ± 0.03 | 17.8 ± 0.96 |
| P1 | 24.2 ± 1.1 | 100.4 ± 3.8 | 0.24 ± 0.01 | 2.42 ± 0.11 | 28.2 ± 1.53 |
| P21 | 23.3 ± 1.1 | 140.5 ± 9.6 | 0.17 ± 0.01 | 1.14 ± 0.33 | 21.7 ± 1.27 |
| P90 | 25.5 ± 0.9 | 119.6 ± 4.5 | 0.21 ± 0.01 | 1.19 ± 0.04 | 31.6 ± 0.64 |
| P180 | 14.1 ± 0.4 | 80.2 ± 2.0 | 0.18 ± 0.01 | 1.05 ± 0.02 | 28.6 ± 1.08 |

Values are means ± SE of 6 to 12 observations at each age (at least one male and one female per litter from three different litters were used), F: fetus, P: postnatal.

TABLE 2
Effect of age on kidney weight and total DNA to body weight ratios and kidney weight, total RNA and protein to DNA ratios in the kidney of pig

| Age (Days) | Kidney Weight/ Body Weight (g/kg) | DNA/ Body Weight (mg/kg) | Kidney Weight/ DNA (g/mg) | RNA/ DNA (mg/mg) | Protein/ DNA (mg/mg) |
|---|---|---|---|---|---|
| F90 | 5.21±0.3 | 53.2±2.7 | 0.10±0.00 | 0.27±0.01 | 5.8 ±0.1 |
| F110 | 3.71±0.1 | 38.5±1.3 | 0.09±0.00 | 0.36±0.00 | 8.2 ±0.4 |
| P1 | 4.78±0.1 | 44.3±1.7 | 0.11±0.00 | 0.56±0.02 | 10.3±0.4 |
| P21 | 3.17±0.1 | 29.6±2.0 | 0.11±0.00 | 0.47±0.03 | 11.3±0.4 |
| P90 | 2.58±0.1 | 16.1±0.5 | 0.16±0.00 | 0.57±0.02 | 19.4±0.6 |
| P180 | 1.36±0.1 | 8.20±0.3 | 0.17±0.00 | 0.58±0.01 | 18.3±0.4 |

Values are means ± SE of 6 to 12 observations at each age (at least one male and one female per litter from 3 different litters were used), F: fetus, P: postnatal.

development in large mammals. In the liver, the highest IGF-I mRNA level and tissue concentration were observed at P21, corresponding to a rapid growth period of this organ. In fact, relatively high ratio of liver DNA / body weight, which is indicative of cell hyperplasia was observed at 3 wk of age. After partial hepatectomy (70%) in adult rats a transient 2-fold increase of IGF-I binding to liver membranes (Santos *et al.*, 1994), supports a role for IGF-I in liver regeneration. In the rat, the liver is believed to be the main source of circulating IGF-I during the postnatal life since liver IGF-I mRNA level increased postnatally (Lund *et al.*, 1986) and parallelled the plasma IGF-I concentration (Donovan *et al.*, 1989). However, the IGF-I mRNA level in the liver peaked at 3 wk of age in the pig which is different from the one observed in the rat. In the pig, serum IGF-I and -II concentrations increased postnatally until 6 mo of age which is in agreement with other observations in pig (Lee *et al.*, 1991 a). During the postnatal life, IGF-II concentration in the liver and kidney reached the maximum value around the suckling period. It seems that IGF-II has a function both in fetuses and in early postnatal life. This is supported by the fact that continuous 14 days infusion of IGF-II increased weight of the kidney and body weight gain as well as efficiency of food conversion in young female rats (Conlon *et al.*, 1995). High liver weight to DNA and total RNA to DNA, which are indicative of cell hypertrophy, are accompanied by high IGFII-R mRNA and IGF-II concentration at birth. An involvement of IGFII-R in liver development is suggested by the observation that receptor number increases in hepatocytes of regenerating liver in rat (Scott and Baxter, 1990). The high tissue concentrations of IGF-I and -II in the kidney (at P1) and IGF-II in the liver (at P1 and P21) with concomitant low mRNA levels of these peptides may be attributed to a regulation at the posttranscription level.

The predominant rat serum binding protein during postnatal life is IGFBP-3 which binds the majority of endogenous IGF-I and -II in a 150 kDa ternary complex composed of IGFBP-3, IGF peptide and an acid-labile protein subunit (Clemmons, 1991). There is a general agreement that plasma IGFBP-3 prolongs the half life of IGFs in the circulation, limits extravascular transit and serves as a reservoir for IGFs (Clemmons, 1991). In the present study, serum IGFs and IGFBP-3 increased postnatally, suggesting that the increase in serum IGFs concentration during postnatal life of pig may be due to the reduced clearance of these peptides. The postnatal increase of serum IGF-I concentration can also be related to postnatal rise in GH specific binding and GHR mRNA in the liver of pig which were observed by Schnoebelen-Combes *et al.*, (1996) and the present study, since GH is the principal hormone stimulating IGF-I production (Copeland *et al.*, 1980). Messenger RNA of IGFBP-1 was the most abundant in the liver which is consistent with the human situation (Han *et al.*, 1996). Liver IGFBP-1 and its mRNA level reached the maximum level at birth and shared a similar pattern to that of serum, implying that liver is a main production site for circulating IGFBP-1. This is supported by the finding that IGFBP-1 is synthesised and released by isolated human hepatocytes (Arany *et al.*, 1994).

Kidney growth in terms of organ weight to body weight and kidney DNA to body weight ratios were relatively high in newborns and accompanied by high IGF-I and -II concentrations. Relatively high levels of IGFI-R and IGFII-R mRNAs were observed in the kidney compared to that of the skeletal muscle, suggesting that kidney may be one of the major target tissues of IGFs. An involvement of IGFs in kidney growth has been proposed based on the observations that IGF-I and IGFBPs are secreted by collecting duct cells (Aron *et al.*, 1991). After unilateral nephrectomy in immature rats, renal IGFs and IGFRs mRNAs as well as specific renal binding of IGF-I to cortical membrane and IGF-II to whole kidney membrane are increased, indicating that IGFs might be involved in kidney regeneration (Mulroney *et al.*, 1992). In the kidney, mRNAs of IGF- I and GHR shared a common developmental pattern which were higher during the fetal life than that of the postnatal life, suggesting that GH may be involved in fetal kidney growth. Marshall *et al.*, (1993) reported that treatment of hypophysectomized rats with GH results in a rapid growth of the kidney and a rise in the renal concentration of IGF-I. The accumulation of mRNAs for GHR and IGF-I are coordinated in the kidney suggesting a direct action of GH to mediate a local production of IGF-I.

Compared with other studied tissues, the skeletal muscle expressed the highest level of IGF-I mRNA which is in agreement with another study in pig (Lee *et al.*, 1993), assuming that the skeletal muscle may be a major source for IGF-I production since it represents the bulk of the body. In the adult rat,

IGF-I mRNA transcripts were found in many tissues, but were the highest in the liver (Murphy *et al.*, 1987), showing a tissue specific regulation of IGF-I mRNA. It was observed that developmental and tissue specific expression of IGF-I mRNA was caused by variation in the 5'-untranslated region (5'-UTR) which could result from the use of different promoters and transcription start sites in the rat IGF-I gene (Hoyt *et al.*, 1988). The IGF-I mRNA level and specific binding of IGF-I in the skeletal muscle increased markedly compared to control after injury, indicating that the locally produced IGF-I is the most likely ligand for IGF-I receptor during muscle regeneration (Jennische and Matejka, 1992). In the present study, GHR mRNA in the skeletal muscle was abundant during the whole developmental period which is consistent with a study in pig (Schnoebelen-Combes *et al.*, 1996). The skeletal muscle exhibited relatively high levels of IGF-I and GHR mRNAs. One can hypothesize that the effect of GH might be mediated by the locally produced IGF-I. However, Coleman *et al.*, (1994) reported that GH administration to pigs did not increase the IGF-I mRNA level in the skeletal muscle. The authors suggested that IGF-I derived from the circulation mediates exogenous GH action on skeletal muscle growth in the pig.

In summary, in the kidney and skeletal muscle, IGF-I, IGFI-R, IGF-II and IGFBP-3 mRNAs were high during the fetal and early postnatal life and decreased thereafter. In the liver, IGF-I mRNA level and tissue concentration were high at 3 wk of age, while in the kidney, IGF-I and -II concentrations were abundant at birth thus corresponding to rapid growth periods of these organs.

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2.3 M. Peng, M.-F. Palin, S. Véronneau, D. LeBel and G. Pelletier. ONTOGENY OF EPIDERMAL GROWTH FACTOR (EGF), EGF RECEPTOR (EGFR) AND BASIC FIBROBLAST GROWTH FACTOR (bFGF) mRNA LEVELS IN PANCREAS, LIVER, KIDNEY AND SKELETAL MUSCLE IN PIG. Domest. Anim. Endocrinol. (Submitted).

2.3.1 Contributions to manuscript 2.3

My contribution to this manuscript was to (1) plan and write the project outline, (2) prepare and organize the sample collection (3) extract total RNA and carry out Northern analysis, (4) provide data to the statistician, (5) prepare figures and write manuscript. We have got technical assistance from Dr. M.-F. Palin and Mr. S. Véronneau for probe preparation. The project and the manuscript preparation were under the direction of Drs. G. Pelletier and D. LeBel.

2.3.2 Manuscript 2.3

# Ontogeny of epidermal growth factor (EGF), EGF receptor (EGFR) and basic fibroblast growth factor (bFGF) mRNA levels in pancreas, liver, kidney and skeletal muscle of pig

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Running head: Developmental EGF, EGFR and bFGF mRNA levels in pig tissues
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**Abstract**

Epidermal growth factor (EGF), EGF receptor (EGFR) and basic fibroblast growth factor (bFGF) mRNA levels were examined by Northern blot analysis in four tissues (pancreas, liver, kidney and skeletal muscle) of pig from fetal 90 days to postnatal 180 days of age. The present study shows for the first time that EGF mRNA increased with advancing age in the kidney and skeletal muscle of pig. A high level of EGF mRNA were observed in the kidney compared to the liver and skeletal muscle. In the pancreas, high levels of EGF mRNA were found in fetuses and newborns and were low in older pigs. Pancreatic EGFR mRNA level parallelled its EGF mRNA whereas in the kidney and skeletal muscle, patterns of EGFR mRNA were reversed to their EGF mRNA levels. In the liver, EGFR mRNA was abundant but EGF mRNA was undetected. In the pancreas and skeletal muscle, the highest levels of bFGF mRNA were found in fetuses of 90 days of age and then decreased with advancing age. In the liver and kidney, there were no major changes in bFGF mRNA levels during the examined developmental periods. These results show that EGF, EGFR and bFGF mRNA levels are developmentally and tissue specifically regulated in pig. In the pancreas, mRNA levels of EGF, EGFR and bFGF were high in fetal and neonatal life and low thereafter. In the kidney and skeletal muscle, EGF mRNA increased with advancing age. EGF may play a role in muscle growth and maintenance in growing pigs during the later stage of development.

## Introduction

In the pig, a wide range of tissues express EGF mRNA including pancreas, liver and kidney, with the highest level in pancreas and kidney with no reference to the age of the animal (1). EGF is known as a regulator in a wide variety of physiological processes including embryogenesis (2), growth (3) tissue repair and regeneration (4). We have shown recently (5) that the level of EGF mRNA is increased in the pancreas of weaning piglets while the organ undergoes rapid growth. EGF receptor (EGFR) is a transmembrane glycoprotein of 170-180 kDa (6). Its tyrosine kinase activity induced by EGF binding is directly implicated in the regulation of cell proliferation (7). High affinity EGF receptors are present in rat pancreatic acini (8) and in human fetal kidney and liver (9). In the regenerating rat liver, the level of EGFR mRNA increases after 1 and 3 days of partial hepatectomy (10). These observations suggest an autocrine action of EGF in various organs.

It was suggested that bFGF may function as "wound hormone", both in the routine maintenance of tissue integrity and during repair after injury (11). Ku *et al.* (12) have shown that following scraping subconfluent bovine aortic endothelial cells, there was a 4- to 10-fold increase in steady state level of bFGF mRNA and protein. Nuclear run-on assay and mRNA stability studies indicate that the increase in steady state bFGF mRNA following scraping is due to the transcriptional activation of the bFGF gene. Also the increase in bFGF mRNA following scraping is dependent on new protein synthesis. Taken together these observations have led to the hypothesis that bFGF may be involved in tissue growth. In our laboratory,

we also observed that other growth factors such as the IGFs and their binding proteins are associated to the organ growth in pig (13).

Despite the importance of EGF and bFGF in regulating proliferation and differentiation of a wide variety of cell types, there is little information on ontogeny of EGF, EGFR and bFGF mRNA levels in tissues of large mammals. The objective of the present study was to examine the levels of mRNA for EGF, EGFR and bFGF in pancreas, liver, kidney and skeletal muscle during development in pig.

## Material and Methods

*Animal and tissue preparation*

Yorkshire purebred fetuses of 90 (F90) and 110 (F110) days of gestation (Normal gestation age is 114 day-old) and pigs of 1 (P1), 21 (P21), 90 (P90) and 180 (P180) days of age were used. For each age two males and two females were obtained from one litter of two different sows. The ages of the fetuses were determined according to the sow's mating date. Pigs, including the sows (with the exception of fetuses), were fasted for 16 h before slaughtering. Animals were slaughtered (stunning and exsanguination) according to recommended code of practice for the care and handling of farm animals, pigs (14). After laparotomy, the uterus was gradually opened and the fetuses were removed and sampled. Immediately after exsanguination, pancreas, liver and kidney were removed by dissection, weighed and

sampled. A sample of the skeletal muscle from the biceps femoris was also taken. Tissue samples of pancreas, liver, kidney and skeletal muscle were frozen in liquid nitrogen and stored at -80°C until analyzed by Northern blot.

*RNA preparation*

Total RNA was extracted according to the method described by Chirgwin *et al.* (15). Tissues were homogenized in 4 M guanidinium thiocyanate using a polytron homogenizer. Supernatants were used for precipitation of RNA in 100 % ethanol and 2 M potassium acetate at -20°C. After centrifugation, the RNA pellets were dissolved in 7 M guanidine hydrochloride. Then, RNA was precipitated again and quantitated by measuring its absorbance at 260 nm.

*Preparation of probes*

A human EGFR complementary RNA (cRNA) probe was transcribed from a 1401 base pairs (bp) *Sma*1-*EcoR*1 cDNA fragment (16), inserted into the pBluescript SK+ plasmid vector. The insert was sequenced using a $^{T7}$Sequencing™ Kit (Pharmacia Biotech Inc, Piscataway, NJ) in order to check the orientation of the cDNA. The template was linearized with *Sma*1 then used in a T7 transcription Riboprobe System (Promega, Madison, WI).

EGF and bFGF probes were prepared by RT-PCR. EGF downstream primer (nt 186-209) 5'-ATCTCTAGAGCGCAGCTCCCACCATTTCAAGTC-3' was designed according to a known region of the pig EGF cDNA sequence (17). The upstream primer 5'-

ATCGGTACCGCATGCTGAAGCCCTCATCACTGG-3' is identical to nucleotide 2607-2630 of the human EGF cDNA (18), a region where sequence is 96% homology for human, rat (19) and mouse EGF (20). A *Kpn*I restriction site for upstream primer and *a Xba*I site for downstream primer were created by addition of nine nucleotides. This primer pair predicts a 917 bp fragment. The bFGF upstream primer 5'-ATCGGTACCTTCAAGGACCCCAAGCGGCTGTAC-3' is identical to nucleotide 107-130 of the marsupial bFGF cDNA sequence (21), while the downstream primer was 5'-ATCTCTAGACCCGTGCCAGTCAGCTCTTAGCAG-3' represents the reverse complement of nucleotides 486-509 of the marsupial cDNA sequence. These primers predict a 421 bp fragment. First-strand cDNA was synthesized from total RNA (6 $\mu$g) using a "SuperScript™ Preamplification System for First Strand cDNA Synthesis" kit (Gibco BRL Life Technologies, Burlington, Ont., Canada) and 500 ng of oligo$(dT)_{12-18}$ primer in a total reaction volume of 30 $\mu$L. An aliquot of 3 $\mu$L was subjected to PCR amplification using a PTC-100™ Programmable Thermal Controller (MJ Research Inc., Watertown, Mass.). The reaction mix contained 3 $\mu$L of reverse transcription mixture, 30 pmol of each primer, 1x PCR buffer (Pharmacia Biotech Inc., Piscataway, NJ), 0.35 mM dNTPs and water to 100 $\mu$L. After overlaying with 3 drops of light mineral oil (Sigma ST. Louis, MO), the reactions were heated to 94°C for 2 min prior to the addition of 4 U of the *Taq* polymerase (Pharmacia Biotech Inc., Piscataway, NJ). The amplification profile comprised 35 cycles: at 94°C for 0.75 min (dissociation), 55°C for 1.25 min (annealing) and 72°C for 2.5 min (extension). The final cycle included a further 5 min at 72°C for complete strand extension.

The PCR amplified cDNA fragments of EGF and bFGF of the predicted length were inserted into the pBluescript KSII+ plasmid vector. The authenticity of the cDNA clones were verified by sequencing using a T7Sequencing™ kit (Pharmacia Biotech Inc., Piscataway, NJ). The templates were linearized with *Eco*RV and then used in a T7 transcription Riboprobe System (Promega, Madison, WI) to synthesis the cRNA probes.

*Northern blot analysis*

The procedure for Northern blot was described by Lehrach *et al.* (22) with some modifications. Briefly, 20 $\mu$g of total RNA was fractionated in a 1 % agarose gel containing 2.2 M formaldehyde before transfer to nylon membranes (Nytran, Schleicher and Schuell, Keene, NH). Membranes were prehybridized for 4 h in a solution of 50 % formamide, 5 × SSC, 50 mM Hepes pH 6.8, 2 mM EDTA pH 8.0, 5 × Denhardt's, 1 % SDS and 200 $\mu$g/ml salmon sperm DNA at 65°C. They were then hybridized in the same solution overnight at 65°C with cRNA riboprobes which were labelled with [$^{32}$P]-labelled dUTP. Membranes were then washed with 0.1 × SSC/ 0.1% SDS twice, 30 min at 85°C and exposed to Kodak films (Eastman Kodak Company, Rochester, NY) with an intensifying screen at -80°C. The size of RNA transcripts was evaluated according to a RNA ladder.

Autoradiograms were scanned using a densitometer (BIO-RAD Imaging Densitometer Model GS-670 Bio-Rad Laboratories Led., Mississauga, Ont., Canada). The relative density of the transcript was expressed as arbitrary optical units. The membranes were reprobed with a 18S ribosomal $^{32}$P-labelled cDNA probe as a control to correct for the possible differences

in RNA loading and transfer in Northern blots. A ratio of the relative density of each specific transcript of growth factors with the relative density of the 18S ribosomal RNA band was calculated before statistical analysis was performed.

*Statistical analysis*

All variables were submitted to an analysis of variance with age, blot and age x blot interaction as main effects in a split plot design using the general linear model procedure of Statistical Analysis System software (23). The different ages were compared using *a priori test* comparisons. The following comparisons were used: (1) fetuses (F90 + F110) vs suckling piglets (P1 + P21) (2) suckling (P1 + P21) vs postweaning (P90 + P180) piglets (3) within fetal ages: F90 vs F110 (4) within suckling ages: P1 vs P21 (5) within postweaning ages: P90 vs P180. Data were considered significantly different if probability value $(P) \leq 0.05$.

## Results

A single EGF mRNA transcript of approximately 4.9 kb was detected in pancreas, kidney and skeletal muscle, with the highest level in the kidney and an undetectable signal in the liver (Fig.1). In the pancreas, EGF mRNA levels were high in fetuses and newborns and relatively low (suckling vs postweaning: $P<0.05$) from P21 to P180 (Fig.2). In kidney and skeletal muscle EGF mRNA levels exhibited an opposite pattern to that of the pancreas (Fig.2). In fact, EGF mRNA was relatively low during the fetal and neonatal life and increased (suckling vs postweaning: $P<0.01$) markedly from 21 and 90 days of age in kidney and skeletal muscle respectively.

A major EGFR mRNA transcript of approximately 10.3 kb was detected in the four studied tissues (Fig. 1). High levels of EGFR mRNA were detected in the fetal and newborn pancreas and decreased ($P<0.001$) abruptly thereafter (Fig. 3) thus paralleling its EGF mRNA levels. In the liver, EGFR mRNA was abundant without any significant change during development (Fig. 3). The kidney and skeletal muscle exhibited relatively high EGFR mRNA levels in fetuses and newborns, then the levels decreased (suckling vs postweaning: $P=0.03$) with advancing age, showing an opposite pattern to their EGF mRNA levels (Fig. 3). A mRNA transcript of 7.0 kb for bFGF was found in the four studied tissues (Fig. 1). In the pancreas and skeletal muscle, the highest bFGF mRNA levels were found in fetuses of 90 days and decreased ($P<0.05$) with advancing age (Fig. 4). In liver and kidney, no effect of age on bFGF mRNA level was observed. There was no effect of blot and age x blot interaction for any growth factors in the studied tissues.

## Discussion

The present study shows, for the first time, that EGF mRNA in the kidney and skeletal muscle increased between three and six months in pig. Messenger RNAs of other growth factors such as IGF-I and -II (24,13) and bFGF in the muscle decreased with advancing age. This suggests that EGF might be an important growth factor regulating growth and maintenance of the skeletal muscle in the later stage of development in pig. It was reported (25) that EGF stimulates protein synthesis in fetal bovine myoblasts. However, in the skeletal muscle and kidney EGFR mRNA decreased with advancing age. Similar observations were made by Aharonov *et al.* (26) who found that EGF and its receptor are regulated in an opposite direction in 3T3 cells. The authors suggested that reduction of EGF receptors by down regulation may be to adjust the cell's subsequent sensitivity to EGF since EGF is still required continuously for mitogenic activity.

The EGF mRNA was more abundant in the pancreas and skeletal muscle than that of the liver which is in agreement with observations that, in pig, EGF mRNA (1) and immunoreactive EGF (27) were mainly present in the kidney and pancreas with no reference to the age of animals. We found that EGF and EGFR mRNA levels present a similar pattern in pig pancreas, suggesting that accumulation of both of them are regulated in a same fashion. High affinity EGF receptors have been detected in isolated rat pancreatic acinar cells (8) and in human pancreatic tissue (28). Combining these data with those of the present study, it appears that EGF may be involved in the regulation of early pancreatic development at the time the pancreas undergoes a rapid growth period in pig (29).

In the pig liver, EGF mRNA was undetected. This is consistent with earlier reports on pig (1) and mouse (30). However, liver EGFR mRNA was relatively abundant during development. It has been reported that EGF has an important developmental effect on liver growth and maintenance (10). The presence of EGF receptors has been shown in human fetal liver and some other tissues (9). It was also found that after 1 and 3 days of partial hepatectomy, EGFR mRNA increased in regenerating rat liver (10). In addition, the concentration of EGF increased in the plasma of rat following partial hepatectomy (31). In mouse, EGF originating from submandibular glands plays a role in promoting early stage of liver regeneration (32). However, in pig, EGF concentration in submaxillary gland has been shown to be very low (27). It was reported that isolated hepatocytes of rat have a great capacity to take up EGF from portal blood (33). Moreover, EGF injected into the portal vein was cleared from the blood through the liver (34), indicating that liver appears to be an important organ which regulates the circulating level of EGF. The presence of EGFR mRNA and the absence of EGF mRNA in the liver, in the present study, suggests that EGF may be involved in liver development through an endocrine action.

The high level of EGF mRNA found in the kidney is in agreement with another study in human (35). A low EGF concentration in plasma compared with the one of urine suggests that urinary EGF could originate from the kidney (36). Immunoreactive EGF in human kidney increased with age (37) in a similar pattern to the EGF mRNA level in pig. Although EGFR mRNA level decreased with advancing age in the kidney, its signal is still higher than pancreas and skeletal muscle during the postnatal life.

Messenger RNA levels for bFGF presented a similar pattern in pancreas and skeletal

muscle, which were high during the fetal life and declined after birth. Basic FGF and its receptors were expressed in human pancreatic cells (38). Messenger RNA of bFGF was also found in mouse skeletal myoblasts (39). In addition, bFGF was shown to be a potent stimulator of DNA synthesis of rat pancreatic acinar cell (40) and of mouse myoblast cells (41). Furthermore, it was suggested that bFGF plays a direct role in promoting skeletal muscle growth, since mechanical loading (stretching) induces bFGF release in the medium of differentiated skeletal muscle culture (42). These findings and the one of the present study, suggest that bFGF might be involved in the pancreas and skeletal muscle development during fetal and early postnatal life of pig.

In summary, our results show that mRNA levels of EGF, EGFR and bFGF in four studied tissues of pig exhibited distinct ontogenic patterns. In the pancreas, EGF, EGFR and bFGF mRNA levels were high in the fetal and neonatal life and low thereafter. In the liver, a high level of EGFR mRNA was observed whereas EGF mRNA was undetected in this organ. In the kidney and skeletal muscle, EGF mRNA levels increased with advancing age. Therefore, EGF may play a role in muscle growth and maintenance in growing pigs during the later stage of development.

Fig. 1. Representive Northern blot of EGF, EGFR and bFGF mRNAs in pig various tissues at 90 days of age (P: pancreas, L: liver, K: kidney, M: muscle). Total RNA was applied to 1% agarose-formaldehyde gel followed by blot hybridization using EGF, EGFR and bFGF RNA probes (at 65°C). Filters were washed twice at 85°C for EGF and EGFR and 65°C for bFGF with 0.1× SSC/0.1% SDS for 30 min. Autoradiographs were proceeded approximately for 1 week for EGF and EGFR and 2 weeks for bFGF respectively at -80°C. The sizes of the transcripts were estimated according to a RNA ladder. The blots were reprobed with a 18S ribosomal $^{32}$P-labelled probe as a control to quantitate RNA loading and transfer.

Fig. 2. Effect of age on EGF mRNA levels in various tissues of pig (F: fetus, P: postnatal). Relative tissue levels of EGF mRNA were evaluated by densitometric scanning of the autoradiograms and were expressed in relative optical units as a ratio of 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Data at each age represented the mean values ± SE obtained from four observations (one male and one female per litter obtained from two different litters).

Fig. 3. Effect of age on EGFR mRNA level in various tissues of pig (F: fetus, P: postnatal). Relative tissue levels of EGFR mRNA were evaluated by densitometric scanning of the autoradiograms and were expressed in relative optical units as a ratio of 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Data at each age represented the mean values ± SE obtained from four observations (one male and one female per litter obtained from two different litters).

Fig. 4. Effect of age on bFGF mRNA levels in various tissues of pig (F: fetus, P: postnatal). Relative tissue levels of bFGF mRNA were evaluated by densitometric scanning of the autoradiograms and were expressed in relative optical units as a ratio of 18S ribosomal RNA signal. One unit is attributed to the maximum ratio and the other values are reported relative to this maximum ratio. Data at each age represented the mean values $\pm$ SE obtained from four observations (one male and one female per litter obtained from two different litters).

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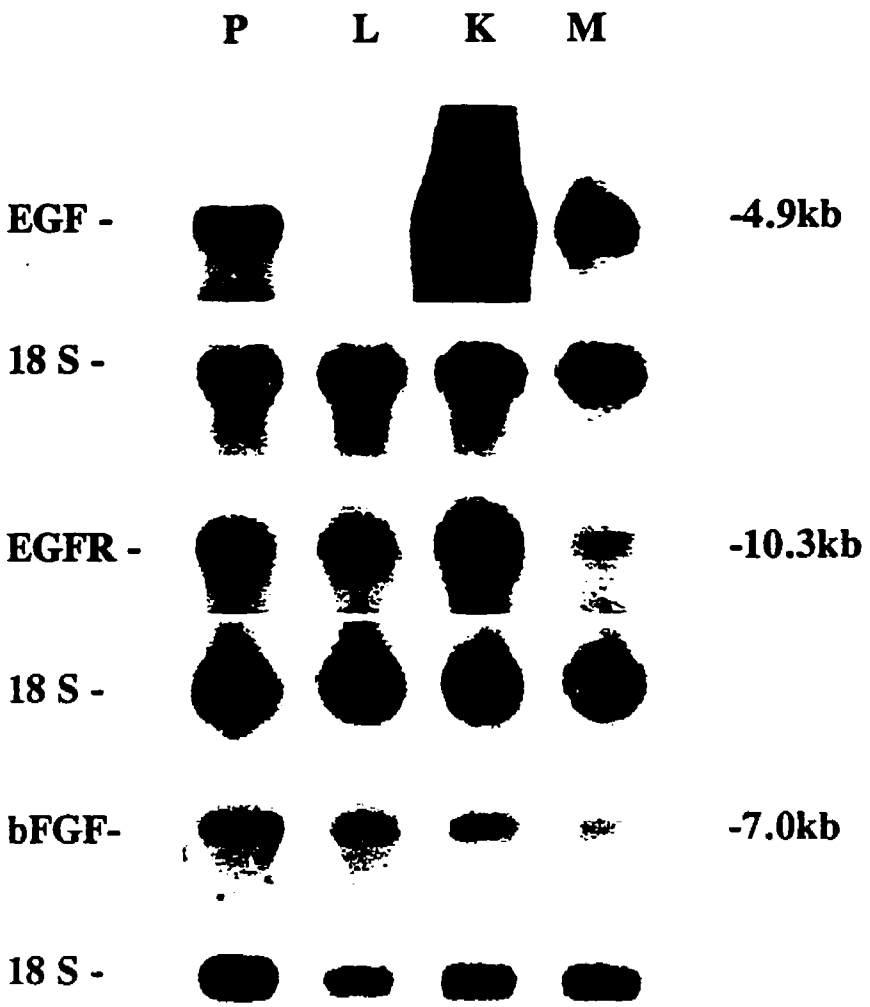



Figure 1.

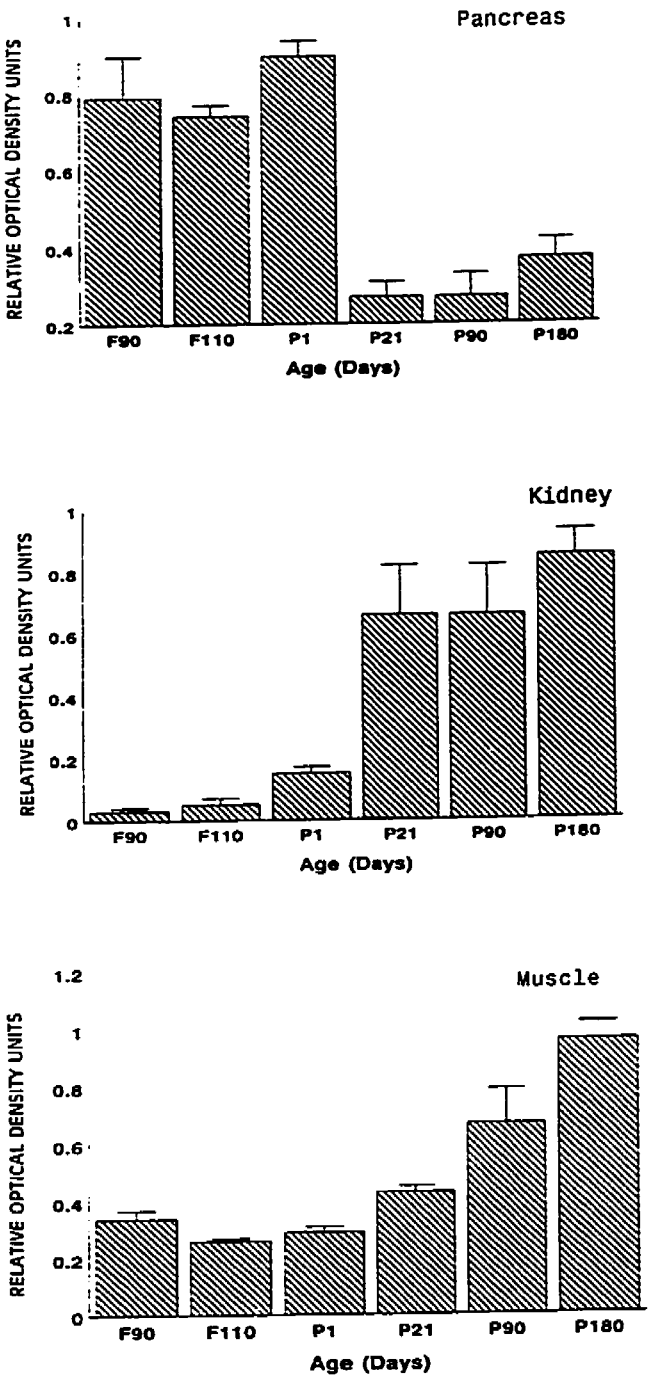
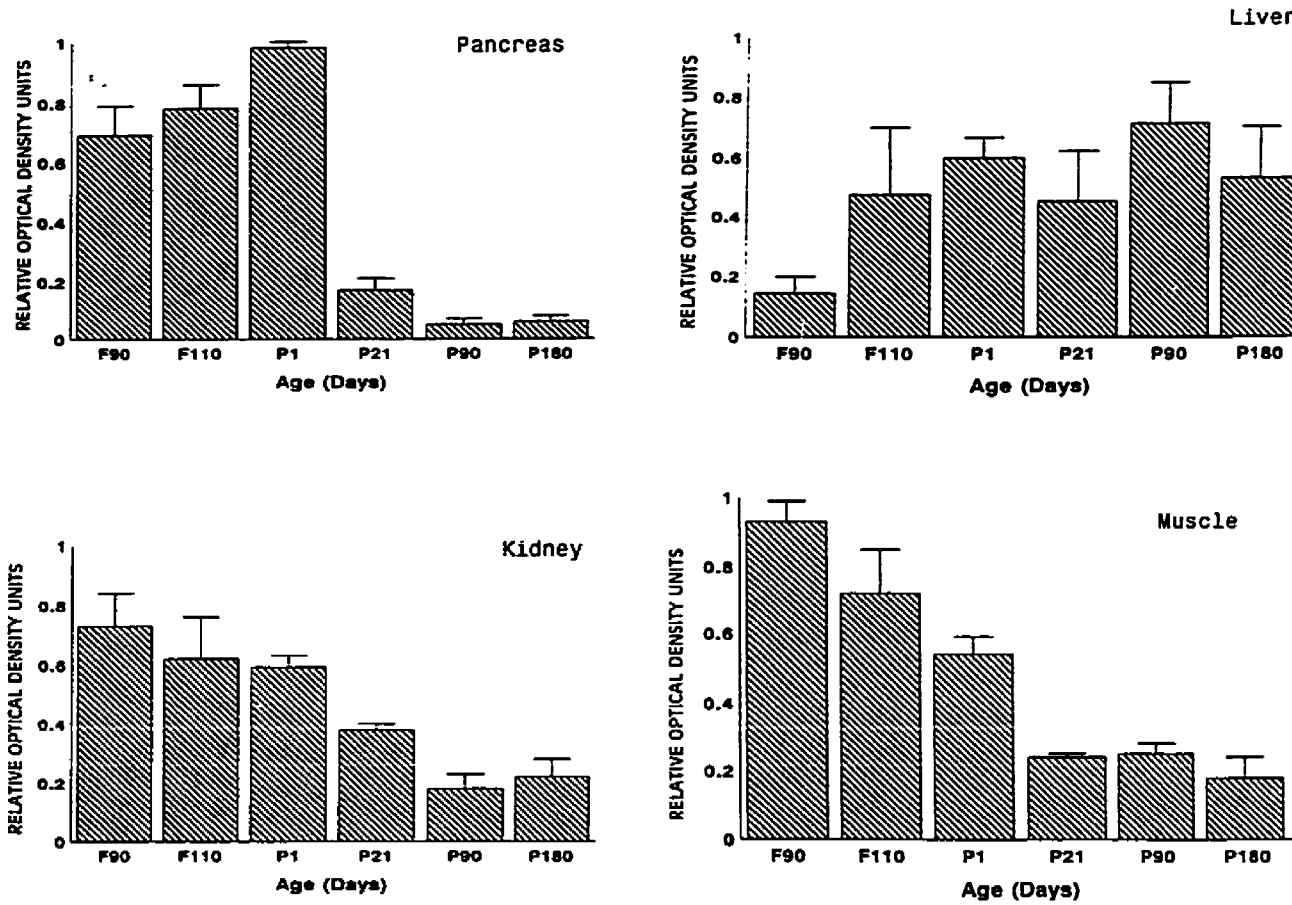

Pancreas
RELATIVE OPTICAL DENSITY UNITS
1
0.8
0.6
0.4
0.2
F90
F110
P1
P21
P90
P180
Age (Days)
Kidney
RELATIVE OPTICAL DENSITY UNITS
1
0.8
0.6
0.4
0.2
0
F90
F110
P1
P21
P90
P180
Age (Days)
Muscle
RELATIVE OPTICAL DENSITY UNITS
1.2
1
0.8
0.6
0.4
0.2
0
F90
F110
P1
P21
P90
P180
Age (Days)

Figure 2.


Pancreas
Liver
Kidney
Muscle
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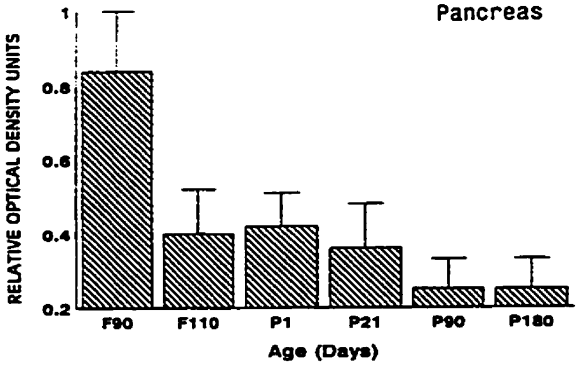
0.8
1
F90
F110
P1
P21
P90
P180
Age (Days)

Figure 3.


Pancreas
RELATIVE OPTICAL DENSITY UNITS
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0.8
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F110
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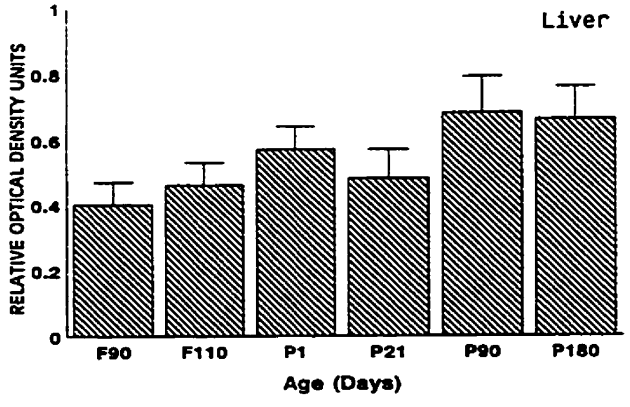
P180
Age (Days)


Liver
RELATIVE OPTICAL DENSITY UNITS
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0.6
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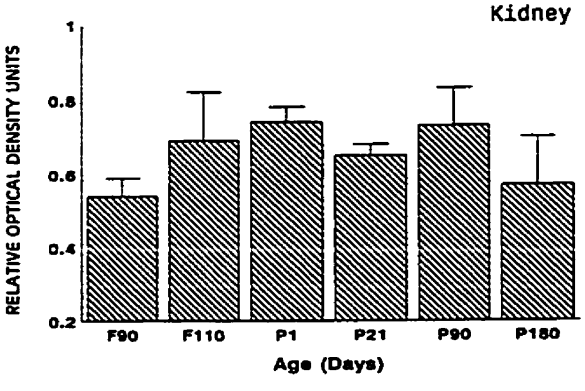
0
F90
F110
P1
P21
P90
P180
Age (Days)


Kidney
RELATIVE OPTICAL DENSITY UNITS
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0.8
0.6
0.4
0.2
F90
F110
P1
P21
P90
P180
Age (Days)

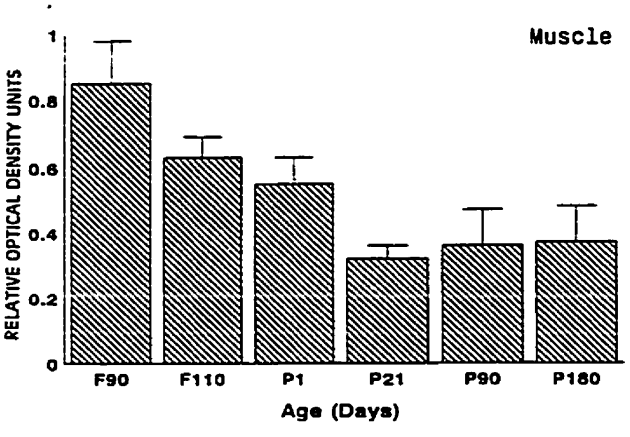
Muscle
RELATIVE OPTICAL DENSITY UNITS
1
0.8
0.6
0.4
0.2
0
F90
F110
P1
P21
P90
P180
Age (Days)

Figure 4.

2.4 M. Peng, M.-F. Palin, D. LeBel and G. Pelletier. EFFECT OF WEANING ON EPIDERMAL GROWTH FACTOR AND ITS RECEPTOR MESSENGER RNA LEVELS IN VARIOUS TISSUES OF PIGLETS. Can. J. Anim. Sci., 1996. 76:621-624.

2.4.1 Contributions to manuscript 2.4

My contribution to this manuscript was to (1) plan and write the project outline, (2) prepare and organize the sample collection, (3) extract total RNA and carry out Northern analysis, (4) determine tissue RNA, DNA and protein concentration, (5) provide data to the statistician, (6) prepare figures and tables and write manuscript. We got technical assistance from Dr. M.-F. Palin for probe preparation. The research project and the manuscript preparation were under the direction of Drs. G. pelletier and D. LeBel.

## 2.4.2 Manuscript of 2.4

*SHORT COMMUNICATION*

# Effect of weaning on epidermal growth factor and its receptor messenger RNA levels in various tissues of piglets

M. Peng,[1] M-F. Palin,[2] D. LeBel[1], and G. Pelletier[2]

[1]*Department of Biology, Faculty of Science, University of Sherbrooke, Sherbrooke, Québec, Canada J1K 2R1;* [2]*Agriculture and Agri-Food Canada, Dairy and Swine Research and Development Centre, Lennoxville, Québec, Canada J1M 1Z3.* *Contribution no. 523, received 13 February 1996, accepted 28 July 1996.*

**Peng, M., Palin, M-F., LeBel, D. and Pelletier, G. 1996. Effect of weaning on epidermal growth factor and its receptor messenger RNA levels in various tissues of piglets. Can. J. Anim. Sci. 76: 621–624. Weights of pancreas, liver, and stomach, and total DNA of pancreas and stomach mucosa per body weight were greater ($P < 0.05$) in weaned piglets than suckling ones at 30 d of age. There was no effect ($P > 0.05$) of weaning on epidermal growth factor (EGF) and EGF receptor (EGFR) messenger RNA levels in liver, stomach, duodenum, and skeletal muscle. Messenger RNAs of EGF in pancreas increased following weaning in piglets of 27 ($P = 0.0001$) and 30 ($P = 0.004$) d of age, indicating a possible involvement of EGF in pancreatic development during this period.**

**Key words:** Weaning, EGF, EGFR mRNA, Pig tissues

**Peng, M., Palin, M-F., LeBel, D. and Pelletier, G. 1996. Influence du sevrage sur les niveaux d'ARN messager du facteur de croissance épidermique et de son récepteur dans divers tissus chez les porcelets. Can. J. Anim. Sci. 76: 621–624. Les poids du pancréas, du foie et de l'estomac et la quantité d'ADN total par poids vif dans le pancréas et la muqueuse stomacale ont été plus élevés ($P < 0.05$) chez les porcelets sevrés que les non-sevrés à l'âge de 30 jours. Aucun effet ($P > 0.05$) du sevrage n'a été observé sur les niveaux d'ARN messagers (m) du facteur de croissance épidermique (EGF) et de son récepteur (EGFR) dans le foie, l'estomac, le duodénum et le muscle squelétique. Les ARNm de l'EGF ont augmenté dans le pancréas à la suite du sevrage chez les porcelets de 27 jours ($P = 0.001$) et de 30 jours ($P = 0.004$), indiquant une implication possible de l'EGF dans le développement du pancréas durant cette période.**

**Mots clés:** Sebrage, EGF, EGFRm RNA, tissus de porc

Development of pancreas around the weaning period is stimulated by dietary changes from milk to solid food (Pierzynowski et al. 1993). It was observed that pancreatic weight and lipase activity were greater in weaned piglets than in suckling ones (Kelly et al. 1991). During the suckling period pancreatic fluid and enzyme secretion remained low (Pierzynowski et al. 1993). Pancreatic juice secretion, output of total protein and activities of various hydrolases (e.g. amylase, trypsin, lipase) increased markedly after weaning (Pierzynowski et al. 1993). However, the mechanisms regulating those changes are not fully understood.

EGF is an important regulator of prenatal and postnatal growth, affecting both mitogenesis and differentiation of a variety of cell types (Carpenter and Wahl 1990). EGF has been shown to be present in various human tissues such as pancreas, stomach and duodenum (Kasselberg et al. 1985). It was reported that livers of fasted rats had 40–50% less EGFR than those of control rats (Freidenberg et al. 1986). Fasting also caused a significant decrease in levels of EGF of gastrointestinal tissues in suckling rats but resulted in minimal changes in adults (Schaudies et al. 1989). Furthermore, levels of immunoreactive EGF decreased in gastrointestinal mucosa of rats fed with a higher proportion of fibre (Schaudies et al. 1991), indicating that dietary fibre either directly or indirectly modulates the content of EGF in the gastrointestinal tract. EGF receptors in the intestinal membranes tended to be higher in weaned piglets than in newborns (Kelly et al. 1992). In fact, no binding sites were detected on the intestinal membranes of suckling piglets. These observations suggest that tissue concentration of EGF is responsive to the nutritional status. One can speculate that at weaning time diet changes may influence EGF status. To our knowledge there is no report on the effect of weaning on EGF mRNA level in mammals. Therefore, the objective of the present study was to determine the effect of weaning on levels of EGF mRNAs and its receptors in pancreas, liver, stomach, duodenum, and skeletal muscle of piglets.

Three Yorkshire purebred sows (each sow having eight piglets) were used. At 21 d of age half of the piglets within the same litter were weaned and the other half stayed with their mother. Creep feed was not provided. At 24, 27, and

**Abbreviations:** EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HGF, hepatocyte growth factor

30 d of age, one suckling and one weaned piglet were slaughtered. Suckling piglets were also slaughtered at 15 and 21 d of age. Milk consumption of suckling piglets was estimated using the weigh-suckling-weigh method (Lewis et al. 1978) on day 21 of lactation. Weaned piglets were removed from the sows and given solid food by gastric intubation (Kelly et al. 1991) two times daily for 3 d before weaning and five times daily for 3 d after weaning. The daily amount of solid food given was 25, 50, 75, 250, 300, and 350 g for 19, 20, 21, 22, 23, and 24-d -old piglets, respectively. The gastric intubation was used to accelerate piglets adaptation to the solid food. It was intended to feed the same level of food for weaned piglets as suckling ones. Faeces were of normal appearance in all pigs during the intubation feeding period and at slaughter there was no indication that digestive processes were in any way impaired. After 24 d of age, the weaned piglets were fed solid food and water ad libitum. Sows were given a meal three times daily, in such a way that the food was completely eaten, in order to prevent suckling piglets from eating the sows' food. Animals were fasted for 16 h and slaughtered (stunning and exsanguination) according to recommended code of practice for the care and handling of farm animals, pigs (Agriculture and Agri-Food Canada 1993). Immediately after exsanguination, pancreas, liver, stomach, duodenum, and skeletal muscle were removed by dissection. The stomach and duodenum were then emptied, rinsed with 0.8% NaCl, and weighted. The mucosa of the stomach and duodenum was scraped off. The mucosa and tissue samples were frozen in liquid nitrogen and stored at –80°C for RNA used for Northern blot and at –20°C for total DNA, RNA, and protein determination. Total DNA, RNA, and protein in the tissues were determined according to the method described previously (De Passillé et al. 1989). Human EGF receptor complementary RNA (cRNA) probes were transcribed from a 1401 base pair (bp) *Sma*1-*EcoR*1 cDNA fragment inserted into the pBluescript SK+ plasmid vector. The templates were linearized with *Sma*1 then used in a T7 transcription Riboprobe System (Promega, Madison, WI). EGF probe was prepared by RT-PCR. EGF downstream primer (5'-ATCTCTAGAGCGCAGCTCCCACCATTTCAAGTC-3') was designed according to known regions of the pig EGF cDNA sequence (Pascall et al. 1991) and represents the reverse complement of nucleotide 186-209 of this sequence. The upstream primer (5'-ATCGGTACCGCATGCT-GAAGCCCTCATCACTGG-3') was identical to nucleotide sequence 2607-2630 of the human EGF cDNA (Bell et al. 1986) in a region where sequence is 96% homologous between human and rat (Saggi et al. 1992). The nine additional nucleotides of each primer include a *Kpn*I restriction site for upstream primer and *Xba*I site for downstream primer. This primer pair predicts a 917 bp fragment. First-strand cDNA was synthesized from total RNA (6 μg) using a "SuperScript™ Preamplification System for First Strand cDNA Synthesis" kit (Gibco BRL, Life Technologies, Burlington, ON) and 500 ng of oligo$(dT)_{12-18}$ primer in a total reaction volume of 30 μL. An aliquot of 3 μL was subjected to PCR amplification using a PTC-100™ Programmable Thermal Controller (MJ Research Inc., Watertown, MA). The amplification profile comprised 35 cycles: at 94°C for 0.75 min (dissociation), 55°C for 1.25 min (annealing) and 72°C for 2.5 min (extension). The final cycle included a further 5 min at 72°C for complete strand extension. Each reaction mix contained 3 μL of the reverse transcription mixture, 30 pmol of each primer, 1x PCR buffer (Pharmacia Biotech Inc., Piscataway, NJ), 0.35 mM dNTPs and water to 100 μL. After overlaying with three drops of light mineral oil (Sigma, ST. Louis, MO), the reactions were heated to 94°C for 2 min prior to the addition of 4 U of *Taq* polymerase (Pharmacia Biotech Inc., Piscataway, NJ). A cDNA fragment of the predicted length was inserted into the pBluescript KSII+ plasmid vector. The template was linearized with *Eco*RV and then used in a T7 transcription Riboprobe System (Promega, Madison, WI) to synthesis the cRNA probe. EGF and EGFR mRNA levels in pancreas, liver, stomach, duodenum, and skeletal muscle were determined by Northern blot. Total RNA, from tissues stored at –80°C, were extracted by the guanidinium isothiocyanate method (Chirgwin et al. 1979). The procedure for Northern blot was described by Lehrach et al. (1977) and Goldberg (1980). Autoradiograms were scanned using a densitometer (BIO-RAD Imaging Densitometer Model GS-670, Bio-Rad Laboratories Ltd. Mississauga, ON). The relative density of the transcripts was expressed as arbitrary optical units. To correct for the possible differences in RNA loading and transfer in Northern blots, a ratio of the relative density of each specific transcript of EGF and EGFR with the relative density of the 18S ribosomal RNA band was calculated before statistical analysis was performed. All variables were submitted to an analysis of variance with weaning and age as main effects in a split-plot design using the general linear model procedure of the SAS Institute, Inc. software (1985). Data were considered significantly different if probability value ($P$) ≤ 0.05.

The average dry matter milk consumption for suckling piglets at 21 d of age was 185.7 g ± 22.1. The daily feed consumption of weaned piglets at 22 d of age was 250 g which can be considered an equivalent amount to the milk intake in term of available nutrients since the digestibility of the solid feed is lower than that of the milk. Body weights were 5.59 ± 0.27 vs. 5.55 ± 0.23, 5.68 ± 0.28 vs. 6.04 ± 0.29, 6.67 ± 0.42 vs. 7.00 ± 0.45, and 7.71 ± 0.63 vs. 7.79 ± 0.68 kg for 21, 24, 27, and 30 d of age weaned vs. suckling piglets, respectively. Pancreas (Table 1), liver and stomach (data not shown) weights per body weight were higher ($P < 0.05$) in weaned piglets than in suckling ones 9 d after weaning (30 d of age). The ratios of total DNA per body weight in pancreas ($P = 0.028$) and stomach ($P = 0.06$), which are indicative of hyperplasia, were also higher in weaned piglets than suckling ones at 30 d of age. In suckling piglets, the ratios of pancreatic weight and total DNA to body weight decreased ($P < 0.05$) whereas ratios of pancreatic weight and RNA to DNA increased ($P < 0.05$) at 30 d of age (Table 1). No age effect ($P > 0.05$) on organ weight and biochemical parameters were observed in liver and stomach of suckling piglets. A single transcript of approximately 4.9 kb for EGF and 10.3 kb for EGFR were detected in all studied tissues with the exception that EGF mRNA

Table 1. Effect of weaning on pancreas weight and total pancreatic DNA to body weight ratios and pancreas weight, total RNA and protein to DNA ratios in piglets (S: suckling, W: weaning)

| Age (d) | Pancreas wt/ Body wt (g kg$^{-1}$) | DNA/ Body wt (mg kg$^{-1}$) | Pancreas wt/ DNA (g mg$^{-1}$) | RNA/ DNA (mg mg$^{-1}$) | Protein/ DNA (mg mg$^{-1}$) |
|---|---|---|---|---|---|
| 15(S)[a] | 0.98 ± 0.06 | 7.00 ± 0.93 | 0.15 ± 0.01 | 1.25 ± 0.04 | 15.8 ± 2.1 |
| 21(S) | 0.97 ± 0.09 | 6.67 ± 0.50 | 0.15 ± 0.01 | 1.08 ± 0.20 | 16.9 ± 2.1 |
| 24(S) | 1.19 ± 0.12 | 7.46 ± 1.63 | 0.17 ± 0.02 | 1.32 ± 0.23 | 20.3 ± 1.6 |
| 24(W) | 1.28 ± 0.22 | 8.82 ± 0.90 | 0.14 ± 0.01 | 1.10 ± 0.10 | 16.8 ± 2.1 |
| 27(S) | 1.24 ± 0.02 | 8.70 ± 1.67 | 0.15 ± 0.02 vs.* | 1.10 ± 0.06 vs.** | 19.7 ± 4.5 |
| 27(W) | 1.55 ± 0.25 | 8.20 ± 1.36 | 0.19 ± 0.01 | 1.78 ± 0.18 | 16.3 ± 2.7 |
| 30(S) | 0.94 ± 0.03 vs.** | 4.83 ± 0.28 vs.* | 0.19 ± 0.01 | 1.57 ± 0.22 | 20.9 ± 1.9 |
| 30(W) | 1.54 ± 0.11 | 8.90 ± 1.16 | 0.17 ± 0.01 | 1.73 ± 0.09 | 19.7 ± 1.5 |

[a] S, suckling; W, weaning.
Values are means ± SE of three observations (obtained from three different litters) at each age. Comparison of suckling vs. weaning. *$P < 0.05$, **$P < 0.01$.

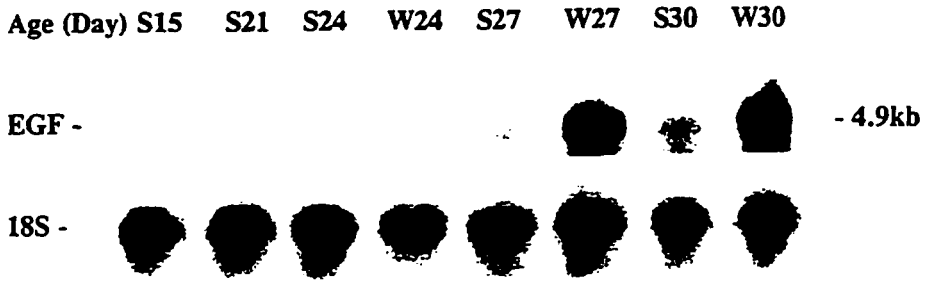


Fig. 1. Effect of weaning on EGF mRNA in pig pancreas. (S, suckling; W, weaning) Twenty μg of total RNA was applied to 1% agarose-formaldehyde gel followed by blot hybridization at 65°C using a [$^{32}$P]-labelled dUTP EGF RNA probe. Filters were washed twice at 85°C with 0.1 × SSC/0.1% SDS for 30 min. Autoradiography proceeded for approximately 1 wk at −80°C. The size of the transcript was estimated according to a RNA ladder. The blots were reprobed with a 18S ribosomal $^{32}$P-labelled probe as a control to quantitate RNA loading and transfer.

was undetected in the liver. An increase ($P < 0.05$) of EGF mRNAs in pancreas was observed in 27- and 30-d-old weaned piglets compared with the suckling ones (Fig. 1). Arbitrary optical density units were 0.91 ± 0.07 vs. 0.23 ± 0.05 at 27 d and 0.73 ± 0.14 vs. 0.31 ± 0.07 at 30 d of age for weaned vs. suckling piglets, respectively. A relatively high level of EGF mRNA was observed in pancreas of 30-d-old weaned pigs, thus corresponding to the significant increase of pancreas weight per body weight in weaned pigs at the same age. No significant change in EGFR mRNA was observed in pancreas following weaning. There was no effect ($P > 0.05$) of weaning on EGF and EGFR mRNAs in liver, stomach, duodenum, and skeletal muscle. Neither EGF nor EGFR mRNA levels were affected ($P > 0.05$) by age in all studied tissues in suckling piglets.

In the present study, liver weight was greater in weaned pigs than in suckling ones, whereas there was no weaning effect on EGF and EGFR mRNAs in this organ. It was reported that 48 h after partial hepatectomy mice treated with EGF showed a rapid increase of DNA synthesis while mice with submandibular glands removed showed a delayed (84 h) response and a slow and sustained increase in DNA synthesis in the liver (Noguchi et al. 1991). This indicates that in mice, EGF derived from the submandibular glands may be involved in liver regeneration. Messenger RNA of EGF was not detected in the liver in the present study, suggesting that during the weaning period liver development may be regulated by EGF derived from endocrine or submandibular glands or regulated by some other growth factors, such as HGF. It was found that exogenous human HGF stimulates DNA synthesis in cultured rat hepatocytes (Fujiwara et al. 1993).

There was no effect of weaning on EGF and EGFR mRNA levels in the stomach. However, the ratios of stomach weight and total DNA of stomach to body weight in weaned piglets were greater than that in suckling ones at

30 d of age. The increase in stomach and pancreas weights in weaned piglets may be due to a change in diet from milk to solid food which affects the anatomy, morphology and function of the gastrointestinal tract in order to adapt to weaning (Kelly et al. 1991).

This is the first report showing that weaning increases EGF mRNA level in the pancreas of mammals. The involvement of EGF in pancreatic growth is supported by the fact that relatively high levels of EGF have been observed in pig pancreatic tissue (Vaughan et al. 1992) and in human pancreatic juice (Hirata et al. 1982). In addition, pancreatic acinar cells have been shown to possess EGF receptors (Korc et al. 1983). Furthermore, EGF increased rates of DNA and protein synthesis in cultured mouse pancreatic acini (Logsdon 1986). In summary, our results and those from previous studies indicate that EGF may be involved in pancreatic development during the weaning period of piglets.

The authors acknowledge J. P. Charuest for statistical analysis and M. Morissette and his team for animal care and tissue sampling at slaughtering. This work was financially supported by the Natural Sciences and Engineering Research Council of Canada grant No. GP 0002887.

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# CHAPTER III

## GENERAL DISCUSSION

In the present study, mRNA levels and tissue concentrations of IGFs and IGFBPs were monitored simultaneously at various developmental stages of the pancreas, liver, kidney and skeletal muscle in pigs. Pig was chosen as a model because of its anatomical and physiological similarities with human. In addition, pig pancreas is big enough to allow all determinations with the same animal. This study shows for the first time that mRNA levels of IGFs, IGFBPs, EGF and bFGF and tissue concentrations of IGFs and IGFBPs are influenced by developmental stages and are sometimes coordinately regulated. It is also the first attempt to make the relationship between mRNA levels, tissue concentrations of IGFs and IGFBPs and organ growth at different physiological developmental stages in mammals. Ontogeny of IGFs mRNA levels have been studied in rat pancreas (Hogg et al., 1993). However, there is no such information reported in pig and no information with regard to IGFs and IGFBPs tissue concentrations either in pig or rat during development. A fast growth period was observed in fetuses of 90 days of age. This growth period was accompanied with high IGF-II mRNA and tissue concentration of the protein. It has been shown that disruption of one of the alleles for the mouse IGF-II gene resulted in a growth-deficient phenotype, implicating a crucial function for IGF-II in fetal growth (de Chiara et al., 1990). More specifically, IGF-II may be important for pancreatic growth since Miettinen et al. (1993) reported that IGF-II mRNA was abundant in both the exocrine and endocrine

human fetal pancreas, exceeding the amount of insulin mRNA. They have also reported that mRNA of IGF-II is under cAMP regulation. Furthermore, blocking the IGF-IR with a monoclonal receptor antibody markedly reduced insulin expression (87 %) and additionally down regulated IGF-II mRNA level (49 %), suggesting that IGF-II is involved in the regulation of pancreatic differentiation and growth through IGF-IR. In the present study, abundant IGF-IIR mRNA was found in the pancreas during fetal life, while IGF-IR mRNA was undetectable. It is assumed that in pig, IGF-II involvement in pancreatic development is mediated through IGF-IR. We have noticed that the IGF-I mRNA levels in pancreas were high during fetal and early postnatal life, which is in agreement with our recent observations in rat (Calvo et al., 1996). However, it displayed an opposite pattern to the one previously reported by Hogg et al. (1993) (Appendix I). The reason for such a discrepancy is unknown, beside the fact that different breeds of rat were used in these studies. These authors have found that both IGF-I and -II stimulate islet cell DNA synthesis, IGF-I being five-fold more potent than IGF-II. The IGF-I receptors were detected in mouse pancreatic acini (Williams et al., 1984) and rat islets (Van Schravendijk et al., 1987), indicating that postnatal pancreas development may be regulated by IGF-I. We found that a high IGF-I tissue concentration at 21 days of age (around weaning period) corresponded to abundant IGF-I mRNA and preceded another fast growth period at 90 days of age. It was reported that during the suckling period, the ratio of pancreas weight to body weight was relatively low in pig (Corring et al., 1978). After weaning (around three to four weeks of age), pancreatic growth and its protein content as well as digestive enzyme activities increased rapidly (Corring et

al., 1978; Kelly et al., 1991), which is in agreement with our observations. In the present study, we did not evaluate changes of the pancreatic weight between 21 to 90 days of age. According to the observations made by Corring et al. (1978) and Kelly et al. (1991), it is possible that a rapid growth of the pancreas occurs before 90 days of age. The high level of IGF-I at 21 days of age indicates its involvement in pancreatic development during the weaning period. This is one of the reasons why we studied the effect of weaning on mRNA levels of IGFs and some other growth factors in gastrointestinal tract development. It has been reported that IGF-I plasma concentrations and mRNA levels in various tissues are regulated by nutritional status (Thissen et al., 1994). Kanamoto et al. (1994) found that expression of c-myc mRNA was greatly enhanced whereas the IGF-I mRNA levels were reduced by consumption of a protein-free diet or by starvation, suggesting that nutrition not only supplies material for body components but also affects signal transduction for growth. During weaning, the diet switches from milk to solid food which might influence growth factors mRNA levels in gastrointestinal organs. However, there is no information about growth factors regulation of organ growth during this period. In order to check wether IGFs and some other growth factors were involved in pancreatic development during the weaning period, we have studied the effect of weaning on mRNA levels of IGFs, IGFRs, IGFBPs, EGF, EGFR and bFGF in pancreas, liver, stomach, duodenum and skeletal muscle of piglets. This study for the first time showed that in the pancreas, EGF mRNA levels increased markedly six and nine days following weaning at 21 days of age. There was no effect of weaning on EGF mRNA levels in any other tissues. In addition, IGFs, IGFRs,

IGFBPs, EGFR and bFGF mRNA levels in all analyzed tissues did not show any change during the weaning period. We have found that the weight of the pancreas and EGF mRNA levels were higher in weaned than that of the suckling piglets at 27 and 30 days of age. The role of EGF on pancreatic development was studied by Logsdon (1986), who found that EGF directly increase DNA synthesis in pancreatic acinar cells in mouse. In addition, high affinity EGF receptors were detected in pancreatic acinar cells (Hormi and Lehy, 1994). Together all these observations support the hypothesis that EGF may participate in the regulation of pancreatic growth during weaning. Further study would be necessary to know whether EGF protein level in the pancreas is altered by weaning. Results of the present study is helpful to understand the relationship between growth factors mRNA expression and organ development during weaning in order to improve piglet performance at weaning. Although IGF-I concentration was high at 21 days of age, there was no effect of weaning on the IGF-I mRNA level in the pancreas. It is possible that an interaction exists between IGF-I and EGF to regulate organ growth during the weaning period. In fact, such an interaction between IGF-I and EGF was observed in 3T3 fibroblasts (Leof et al., 1982). The authors reported that a physiological dose of IGF-I is required for EGF to stimulate 3T3 fibroblasts cells into S phase. Our study shows that in the pancreas, IGFs, IGF-IR, EGF, EGFR and bFGF mRNA levels were all predominant during fetal and early postnatal life, suggesting that multiple growth factors might be involved in pancreatic development.

In the liver, IGF-I mRNA and hepatic IGF-I concentration peaked at 21 days of age which is different from the observation in rat (Appendix I) where IGF-I mRNA increased

postnatally paralleling the plasma IGF-I concentration (Donovan et al., 1989). On the other hand, concentration of IGF-II was predominant during the suckling period (1 day to 21 days of age). The high IGF-I and -II concentrations were accompanied by relatively high ratio of DNA to body weight, an indication of cell hyperplasia and high organ weight to DNA and RNA to DNA, an indication of cell hypertrophy. In addition, serum IGF-I and -II concentrations all increased postnatally, suggesting IGF-II is not only important in fetal growth but also involved in postnatal development. Furthermore, IGF-IIR mRNA was abundant while IGF-IR mRNA was undetected in the liver, which is consistent with the observation in rat (Werner et al., 1989) (Appendix I). Lee et al. (1993) reported that in pig, IGF-I binding capacity was the lowest in liver compared with lung and brain, while IGF-II receptors were identified in all studied tissues. These observations suggest that the action of IGF-II is mediated through IGF-IIR while IGF-I action may be mediated by the insulin receptor in the liver. In the liver, EGF mRNA was undetected whereas EGFR mRNA was abundant during the whole developmental period. It is possible that EGF is involved in liver development through an endocrine action, since rat hepatocytes have a great capacity to take up EGF from the portal blood (Dunn et al., 1986) and that EGF injected into the portal vein is cleared from the blood by the liver (St. Hilaire et al., 1983). Taken together, the literature and our results support the idea that liver development is regulated by the interaction among various growth factors.

In the kidney, IGF-I and -II concentrations peaked at birth and were associated with relatively high rate of kidney growth in terms of organ weight to body weight and total DNA

to body weight. In addition, relatively high levels of IGF-IR and IGF-IIR mRNAs were detected, suggesting that IGFs may regulate kidney growth. Epidermal growth factor is a potent mitogen for cell proliferation (Prigent and Lemoine, 1992). Treatment with EGF of confluent renal proximal tubular cells for 6 consecutive days increases monolayer DNA content 3.3-fold (Nowak and Schnellmann, 1995). In the present study, EGF mRNA levels in the kidney and skeletal muscle increased with age. However, developmental pattern of EGFR mRNA level in these organs was the opposite to that of EGF. Although EGFR mRNA in the kidney decreased with age during the postnatal life, renal EGFR mRNA level was still relatively high compared to the pancreas and skeletal muscle. The high IGFs concentrations and EGF and EGFR mRNA levels in the kidney leads to the hypothesis that these growth factors are involved in kidney development.

In the muscle, EGF mRNA increased with advancing age. Although EGFR mRNA decreased postnatally, the nu mber of EGF receptors might still be significant since skeletal muscle represents the bulk of the body weight. We postulate that EGF may be important for skeletal muscle growth and maintenance in animals of 90 and 180 days of age. In this study, we found that IGF-I mRNA was more abundant in the skeletal muscle compared to the other examined tissues, whereas, in rat, IGF-I mRNA is more abundant in the liver compared to other tissues during the postnatal life (Murphy et al., 1987). It was suggested that developmental and tissue specific expression of IGF-I mRNA is caused by variation in the 5'-untranslated region (5'-UTR) which could result from the use of different promoters and transcription start sites in the rat IGF-I gene (Hoyt et al., 1988). The different developmental

pattern of IGF-I mRNA between rat and pig in pancreas and liver may be due to gene expression regulated differently in pig and rat. We have also observed that IGFs, IGF-IR and bFGF mRNAs were high in fetal skeletal muscle and decreased with advancing age. These results suggest that IGFs, EGF and bFGF are likely physiological regulators of skeletal muscle growth and maintenance in mammals. In our laboratory, we have found that GHR and lipoprotein lipase DNA polymorphism is associated with muscle growth in different lines of landrace purebred pigs. Since EGF mRNA level increased with advancing age in muscle, it is a good candidate to look for DNA polymorphism in order to develop a genetic marker to assist genetic selection of swine for improvement of carcass quality.

In summary, our results indicate that ontogeny of IGFs, IGFBPs, IGFRs, EGF, EGFR and bFGF mRNA patterns are developmentally and tissues specifically regulated in the studied tissues. The fast growth period of pancreas during fetal life may be influenced by IGFs, EGF and bFGF. Pancreatic development during postnatal life may be also regulated by IGF-I. In addition, EGF is an important mitogenic factor involved in pancreatic growth during the weaning period. In the liver and kidney, IGFs concentrations were predominant between 1 and 21 days of age and were associated with rapid growth periods of these organs. In the kidney and skeletal muscle, mRNAs for IGF-I, IGF-IR and IGF-II were high during fetal and early postnatal life, suggesting that these growth factors may participate in organ growth and development. Messenger RNA of EGF in the kidney and skeletal muscle increases with advancing age, supporting the idea that EGF may be involved in growth and maintenance of these organs in pigs.

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ANNEXE

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Original Article

# Development of GP-2 and Five Zymogens in the Fetal and Young Pig: Biochemical and Immunocytochemical Evidence of an Atypical Zymogen Granule Composition in the Fetus[1]

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To uncover the mechanisms involved in the biogenesis of secretory granules, we studied development of the exocrine pancreas in the pig from the fetus up to the mature animal by following the enzyme activities and expression (Northern blot) of five zymogens and GP-2, the major protein of the granule membrane. Fetal pancreas mainly contained chymotrypsinogen and barely detectable amounts of amylase, trypsin, lipase, and elastase. GP-2 was not notably expressed before the Day 21 of life. Ultrastructural examination of the fetal tissue embedded in Epon with osmium postfixation or in Lowicryl at −20°C without postfixation showed dense granules with an irregular shape but also showed that most granules had uncondensed contents, with the aspect of immature granules, or had a dense core surrounded by light material. With immunogold cytochemistry, the concentration of chymotrypsinogen was directly associated with the acquisition of electron density by the granule matrix. These observations suggest that fetal granules have a slower rhythm of zymogen condensation and an irregular shape that could be due to the particular composition of the matrix and the absence of GP-2. We conclude that, in the exocrine pancreas, secretory granules can be formed under various conditions, even with a matrix containing a ratio of components very different from that of the normal mature animal. (*J Histochem Cytochem 44:481–499, 1996*)

KEY WORDS: Secretion granule; *Trans*-Golgi network; Exocrine pancreas; Regulated secretion; Electron microscopy; Immunocytochemistry; Pig.

## APPENDIX I

Comparison of IGFs and IGFBPs mRNA levels in rat and pig during fetal and postnatal life

<table>
<tr><th rowspan="2">mRNA levels</th><th rowspan="2">Periods</th><th colspan="2">Pancreas</th><th colspan="2">Liver</th></tr>
<tr><th>Rat</th><th>Pig</th><th>Rat</th><th>Pig</th></tr>
<tr><td rowspan="2">IGF-I</td><td>Fetal</td><td>High</td><td>High</td><td>Low</td><td rowspan="2">Peaked at P21</td></tr>
<tr><td>Postnatal</td><td>Low</td><td>Low</td><td>High</td></tr>
<tr><td rowspan="2">IGF-II</td><td>Fetal</td><td>High</td><td>High</td><td>High</td><td>High</td></tr>
<tr><td>Postnatal</td><td>Low</td><td>Low</td><td>Low</td><td>Low</td></tr>
<tr><td rowspan="2">IGFBP-1</td><td>Fetal</td><td rowspan="2">Peaked at P21</td><td rowspan="2">barely detectable</td><td>High</td><td rowspan="2">Peaked at P1</td></tr>
<tr><td>Postnatal</td><td>Low</td></tr>
<tr><td rowspan="2">IGFBP-3</td><td>Fetal</td><td rowspan="2">No change</td><td>High</td><td>Low</td><td rowspan="2">No change</td></tr>
<tr><td>Postnatal</td><td>Low</td><td>High</td></tr>
<tr><td rowspan="2">IGFI-R</td><td>Fetal</td><td rowspan="4">Not determined</td><td>High</td><td colspan="2" rowspan="2">Undetectable</td></tr>
<tr><td>Postnatal</td><td>Low</td></tr>
<tr><td rowspan="2">IGFII-R</td><td>Fetal</td><td rowspan="2">Undetectable</td><td>High</td><td rowspan="2">No change</td></tr>
<tr><td>Postnatal</td><td>Low</td></tr>
</table>

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Present study manuscripts 2.1 and 2.2

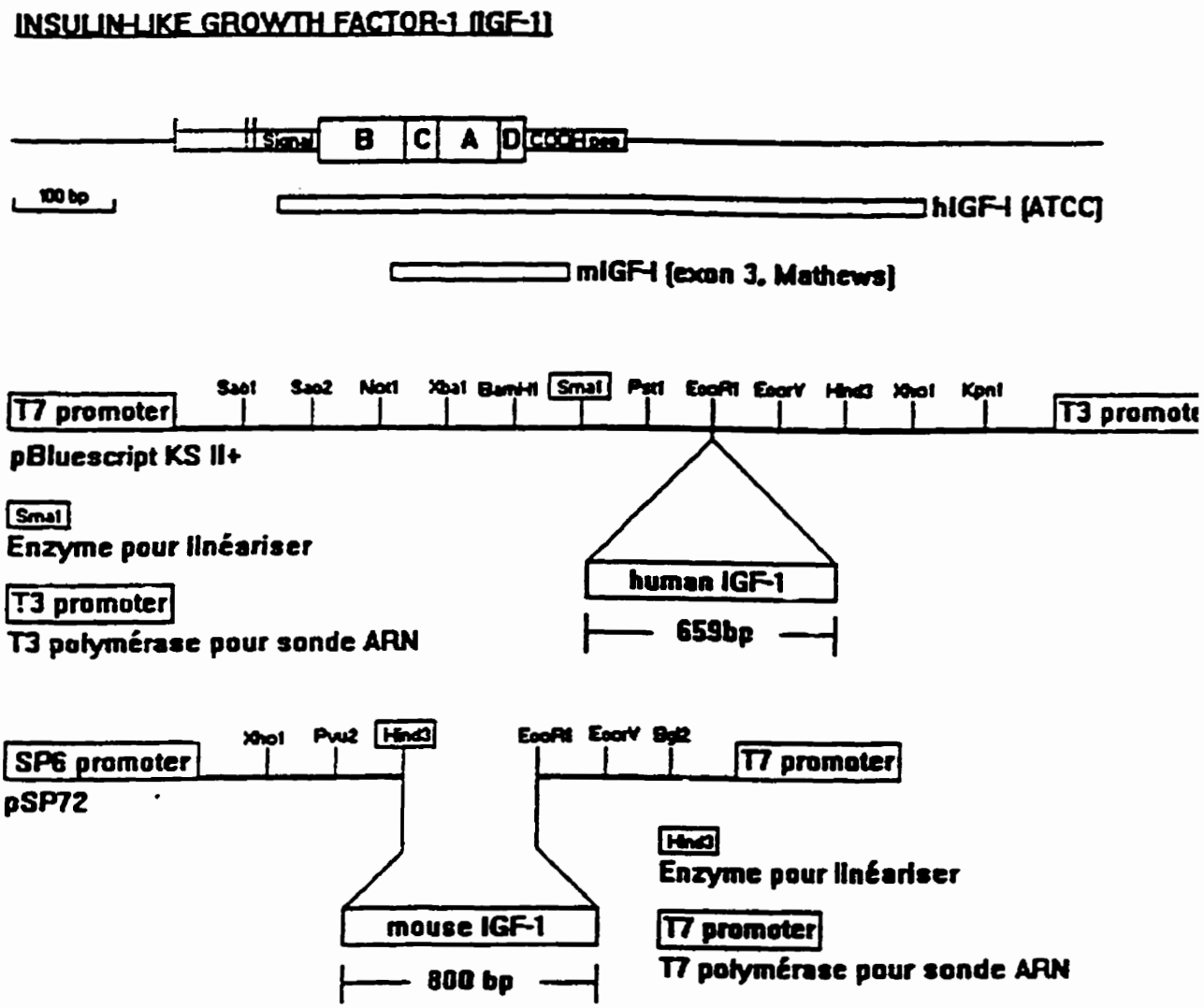
## APPENDIX II

Body weight and food consumption of piglets around the weaning period

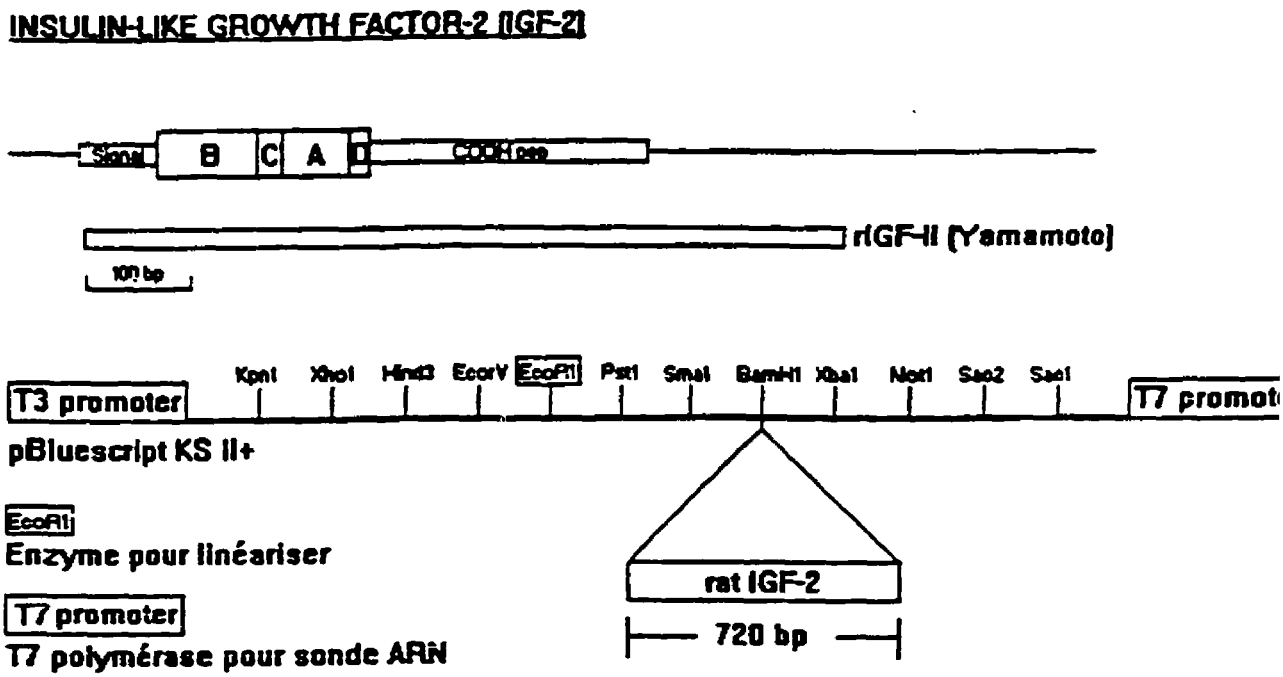
| Age (Days) | 21 | 24 | 27 | 30 |
|---|---|---|---|---|
| Piglets | | Body weight (g) / Piglet | | |
| Suckling | 5546±229.9 | 6045±289.9 | 7002±454.2 | 7793±684.1 |
| Weaned | 5587±274.2 | 5678±284.1 | 6674±416.5 | 7708±628.1 |
| n | 9 | 9 | 6 | 3 |
| Period (Days) | 14-21 | 21-24 | 24-27 | 27-30 |
| Piglets | | Average daily body weight gain (g) / Piglet | | |
| Suckling | 201.9±27.3 | 156.1±45.7 | 243.6±34.1 | 133.9±92.8 |
| Weaned | 238.6±20.9 | 24.7 ±38.5 | 282.2±65.6 | 244.4±100.9 |
| n | 9 | 9 | 6 | 3 |
| Period (Days) | 19-21 | 22-24 | 25-27 | 28-30 |
| | | Daily dry matter consumption (g) / Piglet | | |
| Weaned piglets | 50 | 300 | 312.5±44.1 | 410.6±68.1 |
| | | Daily dry matter consumption / Body weight (%) | | |
| Weaned piglets | 0.9 | 5.0 | 4.7 | 5.3 |

Daily milk consumption for suckling piglets was estimated using the weigh-suckling-weigh method. Daily milk dry matter consumption of suckling piglets at 21 days of age was 185.7 ± 22.1 g/piglet (n=6) (The dry mater content of the milk and the dry feed were 18% and 90%, respectively). The commercial name of the dry feed was Poupon which is produced by Coopérative Fédérée de Québec, Ste-Rosalie, Québec. The milk dry matter consumption / body weight of suckling piglets at 21 days of age was 3.3%.

# APPENDIX III

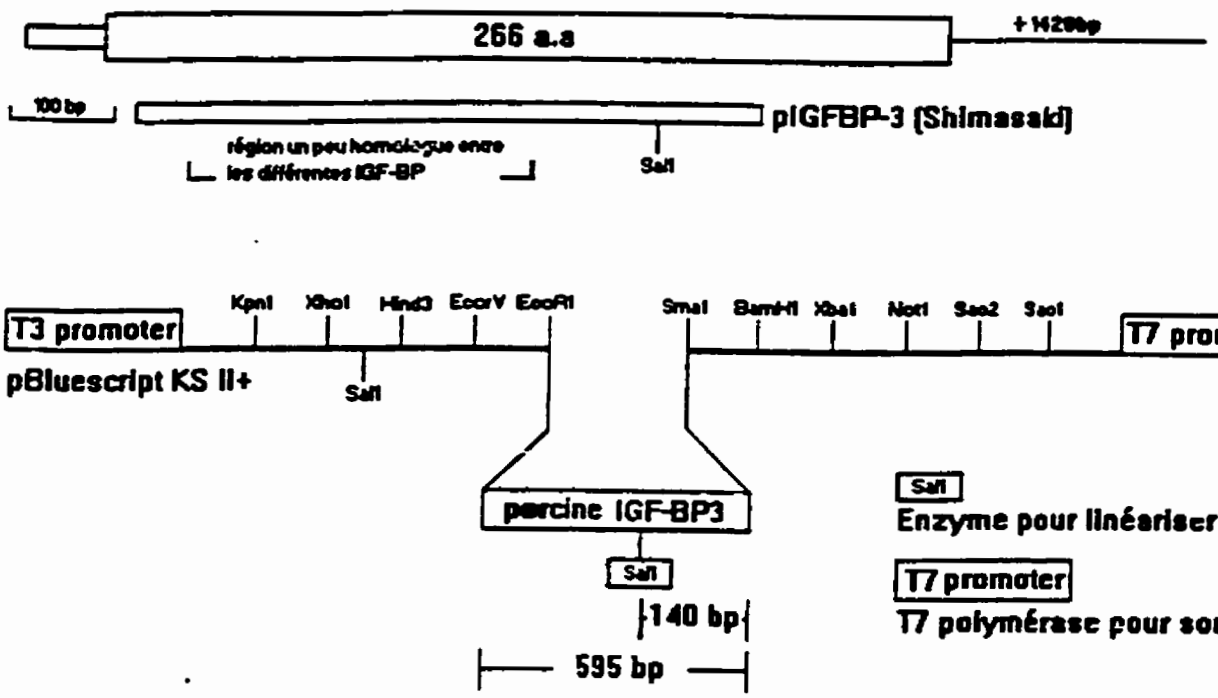


## APPENDIX IV

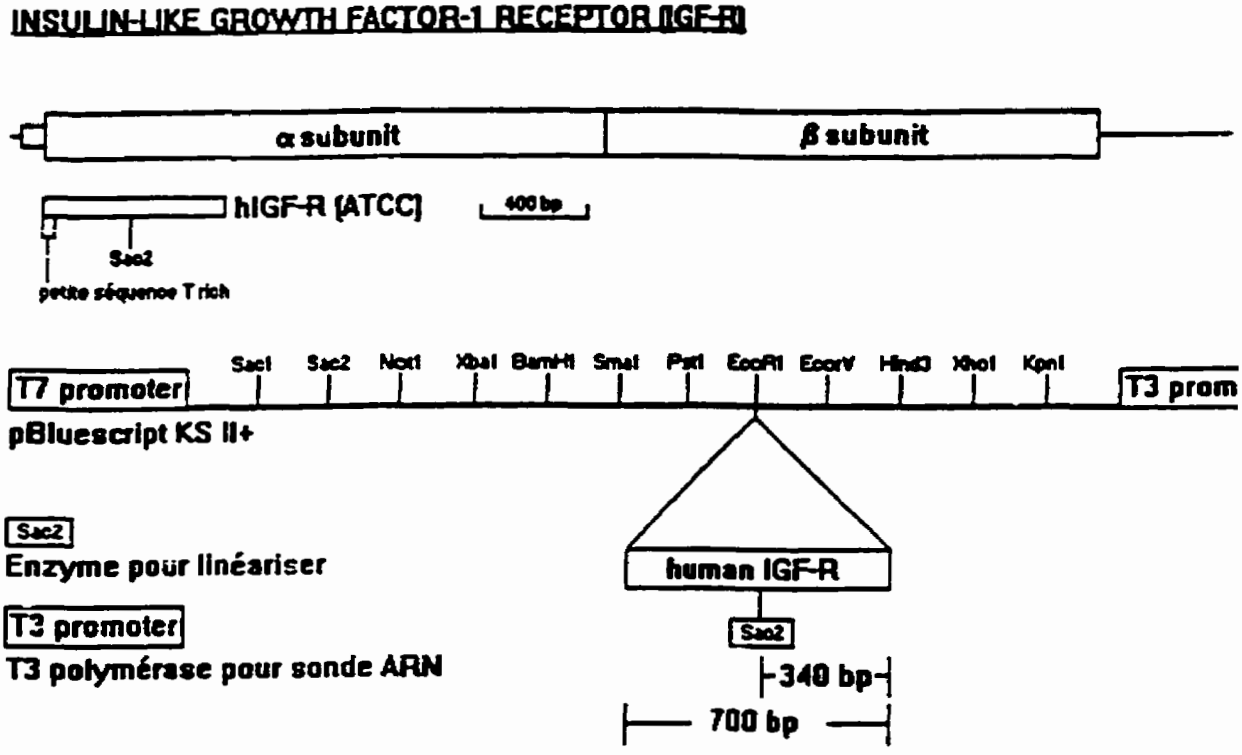

INSULIN-LIKE GROWTH FACTOR-2 (IGF-2)
Signal
B
C
A
D
COOH pep
rIGF-II (Yamamoto)
100 bp
T3 promoter
Kpn1
Xho1
Hind3
EcorV
EcoR1
Pst1
Sma1
BamH1
Xba1
Not1
Sac2
Sac1
T7 promot
pBluescript KS II+
EcoR1
Enzyme pour linéariser
T7 promoter
T7 polymérase pour sonde ARN
rat IGF-2
720 bp

## APPENDIX V

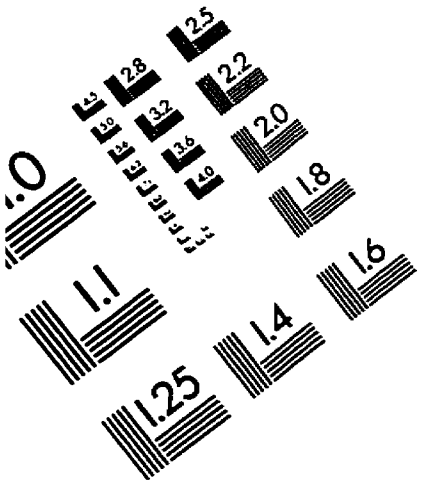
**INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 [IGF-BP3]**

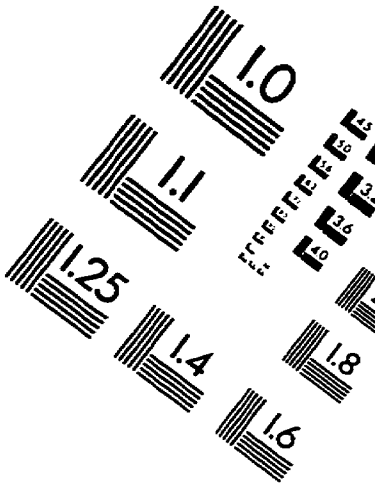


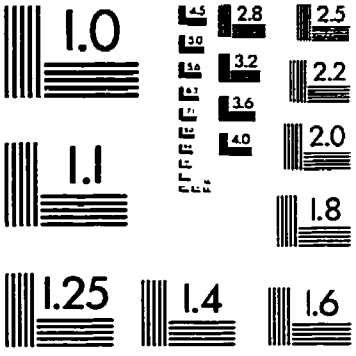
## APPENDIX VI

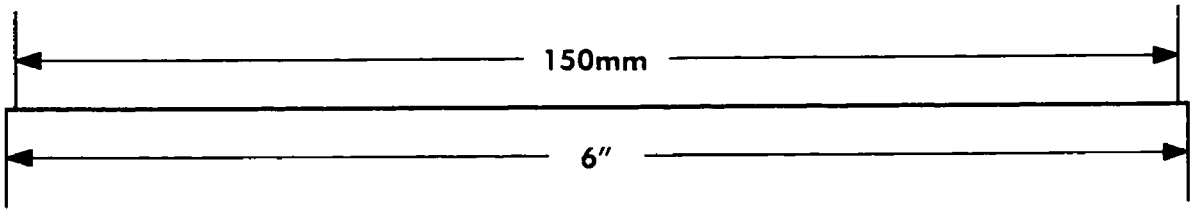

INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR (IGF-R)
α subunit
β subunit
hIGF-R (ATCC)
400 bp
Sac2
petite séquence T rich
Sac1 Sac2 Not1 Xba1 BamH1 Sma1 Pst1 EcoR1 EcorV Hind3 Xho1 Kpn1
T7 promoter
T3 prom
pBluescript KS II+
Sac2
Enzyme pour linéariser
T3 promoter
T3 polymérase pour sonde ARN
human IGF-R
Sac2
340 bp
700 bp

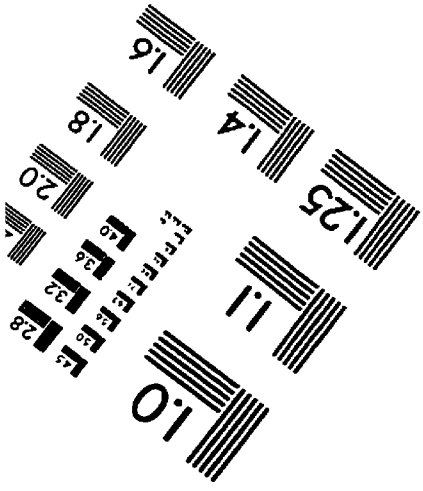
# IMAGE EVALUATION TEST TARGET (QA-3)

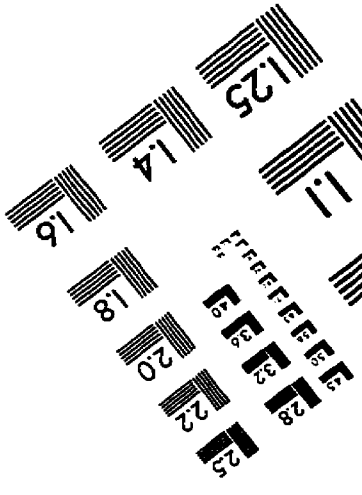
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